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Evaluation of tulathromycin as an antimicrobial therapy in the caprine species

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Evaluation of tulathromycin as an antimicrobial therapy in the caprine species

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ABSTRACT

The number of antimicrobials available to treat bacterial respiratory disease in goats is extremely limited. Currently, only ceftiofur has been approved for use in this species. Concerns over the development of antimicrobial resistance in bacteria that infect humans have led to proposed restrictions on the use of this drug in food-producing animals. Tulathromycin, a triamilide macrolide antibiotic found to be safe and effective against respiratory bacterial pathogens in cattle and swine, was formulated to enhance persistence in lung tissue. The Minor Use Animal Drug Program (National Research Support Project-7) identifies and supports research directed at meeting the Food and Drug Administration (FDA) requirements for drug approval in minor species or for minor uses in major species. The current work was undertaken to support FDA approval of tulathromycin in the caprine species by providing an assessment of target animal drug safety, drug tissue elimination, and drug efficacy. Tulathromycin had no detrimental clinical effects, even in animals treated with 5X the proposed label dose for three times the proposed label duration. Tissue elimination and pharmacokinetic behavior paralleled that found in both cattle and swine. Efficacy analysis was conducted on the clinical effect and pharmacokinetic behavior of tulathromycin in goats as well as *in vitro* antimicrobial effects on bacterial isolates recovered from goats. Bacteria isolated from goats with clinical cases of pneumonia were highly susceptible to this drug in several *in vitro* assays. Overall, this research demonstrated that tulathromycin would be a safe, effective, and valuable medication in the treatment of bacterial pneumonia in goats.

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Goats (*Capra aegagrus hircus)* are an important small ruminant species both in the United States and around the world. The Food and Agriculture Organization of the United Nations estimates that globally over four million pounds of goat meat and 15 million pounds of goat milk are sold annually; these values underestimate true production since many goat products are consumed locally (http://faostat.fao.org/site/569/default.aspx#ancor, 7-13-10). The National Agricultural Statistics Service of the USDA estimated goat numbers in the United States as of January, 2010, at 3.04 million head, with over 2.5 million of these being meat goats.[1] While these numbers are small compared to other livestock species (93.7 million cattle and 66.8 million swine), the caprine species represents an important segment of U.S. agriculture. In addition to providing food and fiber products, goats are frequently used for additional activities such as brush control. As browsers they are fit to survive on plant material that may be ignored by other ruminant species, and their use in vegetation management and to control noxious plants represents a major contribution of this species (Debeuf *et al*., 2004; Glimp, 1995). Although meat goat production is rarely the sole source of income in a livestock enterprise, it can contribute to the overall economic viability of these operations.

While goat production is a growing segment of U.S. agriculture, the number of FDA-approved medications available to treat this species is severely limited (Fajt, 2001; Fajt,

[1] USDA Economics, Statistics, and Marketing Information System, National Agricultural Statistics Service Report, July 24, 2009 and Jan 28, 2000.

2003; Webb *et al*., 2004). The approval process for a new animal medication has very specific requirements. The time to complete this process had been estimated at 8-10 years and the cost at $20-40 million; therefore, it is not cost effective for pharmaceutical companies to pursue these requirements for minor species use (United States Department of Agriculture National Research Support Project 7, 2007). Consequently, many drugs are administered to goats in an extra-label manner with no scientific information on drug behavior, potential toxicity, and adequate withdrawal periods for drug removal from products marketed for human consumption (Berge *et al*., 2006; Fajt, 2001; Fajt, 2003).

Bacterial pneumonia is a frequent health problem in small ruminants (Ackermann & Brogden, 2000; Washburn *et al*., 2007; Yener *et al*., 2009). *Mannheimia haemolytica*, *Bibersteinia (Pasteurella) trehalosi*, *Pasteurella multocida*, and *Mycoplasma* spp. are the most frequently identified bacterial pathogens in goats with clinical respiratory disease (Berge *et al*., 2006; Brogden *et al*., 1998; Washburn *et al*., 2007; Yener *et al*., 2009; Zamri-Saad & Mera, 2001). Currently the cephalosporin ceftiofur is the only antimicrobial approved for use in goats (Fajt, 2001; Fajt, 2003; Washburn *et al*., 2007). Several limitations exist with use of this antibiotic, including the need for daily administration to maintain therapeutic concentrations and the lack of efficacy associated with Mycoplasma infections (Rosenbusch *et al*., 2005). Concerns over antimicrobial resistance patterns in human bacterial isolates and their potential association with antibiotic use in food animal species had led to potential legislative restrictions that may be placed on use of this drug class in livestock (Nolen, 2009). Such restrictions would remove the only drug approved for use in goats.

Tulathromycin is a novel triamilide in the macrolide class shown to be safe and effective against bacterial respiratory pathogens in cattle and swine (Benchaoui *et al*., 2004; Evans, 2005; Hart *et al*., 2006; Nowakowski *et al*., 2004). Its unique structural properties permit rapid dissemination out of plasma and extended residence time in tissues, primarily the lung (Benchaoui *et al*., 2004; Evans, 2005; Nowakowski *et al*., 2004). Since goats are susceptible to many of the same bacterial pathogens as cattle, tulathromycin may prove useful in the caprine species.

The primary objective of this work was to assess tulathromycin as a therapeutic agent in goats with the intent to partially meet FDA/CVM requirements for a label claim in this species. All studies were conducted according to Good Laboratory Practice standards (21 CFR Part 58). Studies were monitored by a Food and Drug Administration (FDA) representative to ensure adherence to GLP standards. The individual studies were conducted to address the safety of tulathromycin in goats; to assess the tissue depletion of tulathromycin in goats to provide data useful for establishing an appropriate withdrawal time for this drug in potential food products; and to utilize a method of establishing efficacy of tulathromycin against major respiratory pathogens by studying pharmacokinetic and pharmacodynamic behavior of this drug in goats.

DISSERTATION ORGANIZATION

This dissertation is organized into six chapters including four separate scientific manuscripts. Chapter 1 provides a brief introduction on the reasons for conducting the present research, an overview of the dissertation format and a literature review with background information describing the reasons for conducting this research. References

cited in the general introduction and the literature review are listed at the end of Chapter 1 and formatted to the style of the *Journal of Veterinary Pharmacology and Therapeutics.* Chapter 2 reports on the assessment of the safety of tulathromycin administration in goats and evaluated animals at the label dose, 3 times the label dose, and 5 times the label dose for three times the recommended duration of therapy for cattle and swine. This manuscript was published in *Journal of Veterinary Pharmacology and Therapeutics*. Tissue residue evaluation and an estimation of tulathromycin content in lung (the target tissue) are covered in Chapter 3 in order to provide data to FDA for the establishment of a withdrawal time for tulathromycin in goats. This manuscript is under review by the co-authors of the Food Animal Residue Avoidance Databank (FARAD) and will be submitted to the *Journal of Veterinary Pharmacology and Therapeutics.* In Chapter 4, pharmacokinetic behavior and modeling assessment of tulathromycin was conducted on three study groups of goats. This paper has been accepted with revisions by the *Journal of Veterinary Pharmacology and Therapeutics.* Chapter 5 contains a review of MIC values for bacterial isolates collected from diseased tissues in goats with clinical pneumonia. The purpose of this evaluation was to assess susceptibility to tulathromycin although a panel of antimicrobial drugs was used. In addition, minimum bactericidal concentration (MBC) assessments and kinetic killing assays were performed on a subset of isolates to further demonstrate bacterial sensitivity to tulathromycin. This manuscript will be submitted to the *Journal of the American Veterinary Medical Association.* Chapter 6 has some final conclusions and implications of the research that may lead to the ultimate goal of FDA approval of this drug in caprines.

LITERATURE REVIEW

Industry background

The domestic goat (*Capra aegagrus hircus)* has many characteristics that make it a valuable livestock species. As primary browsers, goats can survive on range land and plant species that other ruminants cannot. According to the Food and Agriculture Organization of the United Nations (http://faostat.fao.org/site/339/default.aspx, 6-25-10), goat meat is the most consumed meat in the world, and the world's goat population is over 861,000,000 head. In the United States, goats are utilized for production of meat, milk and milk products, hair and fiber products, for brush control, and as companion animals (Glimp, 1995). Ethnic and faith-based groups that consume goat products have increased 100% in this country over the last 10 years, and the demand for goat products to supply these niche markets has increased as well (Solaiman, 2007). The Hispanic population alone increased 57.9% from 1990 to 2000, accompanied by increases in Asian, African, and Muslim populations. In 2005, the United States was the number one importer of goat products in the world, accounting for 18.2% of the total market share. Nearly 25 million pounds of goat meat were imported into this country in 2006 (Solaiman, 2007). From 1997 to 2002, the number of U.S. farms selling goats increased 145.3% from 29,237 to 43,495, demonstrating expansion of U.S. goat production in response to this increasing demand (National Agricultural Statistics Service, USDA, 2010). While goats represent a growing segment of agriculture, their numbers are far lower than those of other livestock species and many of their health issues cannot be addressed with FDA-approved medications.

Food and Drug Administration Background

The Food and Drug Administration Center for Veterinary Medicine (FDA CVM) governs the evaluation and approval of medications to be used in animals. Initially the investigator conducting the research must receive FDA approval to administer the drug for experimental purposes under the Investigational New Animal Drug (INAD) exemption. The research must be conducted under Good Laboratory Practices (Code of Federal Regulations Title 21, 2009). A New Animal Drug Application (NADA) review assesses data in three main areas. A safety evaluation is conducted in the target species to ensure there are no adverse effects associated with its administration. A human food safety study that evaluates drug elimination in specific tissues must be performed in animals that produce products which could be consumed by humans. Finally, the drug must be shown to be effective against the condition for the label claim addressed in the application. (http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/NewAnimal-DrugApplications/ucm146121.htm, 3/25/10). In addition, studies on bioavailability, injection site tolerance, oral toxicity in laboratory animals, effects on fetal tissues in laboratory animals, microbial mutation rates, mammalian cell line cytotoxicity, radiotracer tissue depletion, comparison metabolism, microbial food safety, and induction of antimicrobial resistance transfer may also be components of an NADA portfolio (NADA, 141-244, 2005).

Investigations designed to meet these requirements can be costly and difficult to accomplish with no guarantee to the pharmaceutical company of any return on investment. Estimates for drug approval on a new medication can reach as high as $40 million and on the

addition of a label claim for an existing medication as high as $10 million (United States Department of Agriculture National Research Support Project 7, 2008). Since each medication must be shown to adequately meet these requirements for an individual species and a specific condition, potential sales must provide sufficient incentive to a pharmaceutical company to justify the financial and time outlay for this process. Consequently, very few medications are labeled for use in individual species such as goats that do not represent a major segment of U.S. agriculture.

Species for which there are limited labeled medications often must be treated in an "extra-label" manner. The FDA recognized that use of medications in this manner could result in unsafe consequences for animals being treated in addition to potential residues entering the human food chain. In 1976, the FDA formed a committee to address needs for medications to treat less populous species or uncommon conditions. The IR-4 Animal Drug Program established in 1982 formed a partnership between animal industries and the FDA to identify and secure drug approvals for minor species (United States Department of Agriculture National Research Support Project7, 2007).

The FDA CVM-defined major species are cattle, swine, horses, dogs, cats, and chickens, and turkeys (Fait, 2003; NADA, 141-244, 2005). Species not included in this group are classified as "minor" because their numbers and industries are smaller than those of major species. USDA estimates gross annual income from agricultural production of minor species at $9 billion per year (NADA, 141-244, 2005). While the economic contribution from these industries is large as a whole, contributions from individual species for specific diseases in these groups frequently do not justify the outlay necessary to meet FDA medication approval.

By comparison, annual income from major U.S. agricultural industries are estimated at $76 billion from cattle production, $16 billion from swine production, and $32 billion from poultry production (United States Department of Agriculture Economic Research Service, 2010).

The Animal Medicinal Drug Use Clarification Act (AMDUCA) enacted in 1994 provided veterinarians with the authority to prescribe medications "off-label" under a valid veterinary-client-patient relationship in species or for conditions that do not have approved medications (Berge *et al.*, 2006; Fajt, 2001; Fajt, 2003; Webb *et al.*, 2004). There are many restrictions in this directive, including the requirement for all prescribed drugs to be FDA-approved in at least one other species. Certain drug classes are expressly prohibited from off-label use, and the utilization in minor species of feed additives and medications applied specifically to increase production is not permitted under AMDUCA. Additionally, veterinarians who prescribe suitable medications are responsible for determining the appropriate dose for the species being treated and establishing an appropriate withdrawal period to prevent violative residues in potential human food products (Fajt, 2001; Fajt, 2003). Medications approved for one species may not be appropriate for extra-label use in another species. For example, the macrolide antibiotic tilmicosin is labelled for treatment of bacterial pneumonia in cattle and has been shown to be safe and effective in the treatment of respiratory disease in sheep; however, this medication may be cardiotoxic and potentially fatal when administered to goats (Christodoulopoulos *et al*., 2002; Jordan *et al*., 1993). Data from controlled studies in the target species is invaluable to assist clinicians in the choice of a safe and effective therapeutic

that fulfills AMDUCA requirements and avoids entry of drug residues into the human food chain.

Minor Species and Minor Use Program

The Minor Use and Minor Species Animal Health Act of 2004 amended the Food, Drug, and Cosmetic Act to help facilitate the development of drugs for less common species and indications (United States Department of Agriculture National Research Support Project7, 2007). It provides additional classifications for these compounds that assist pharmaceutical companies in receiving drug approval with lower financial outlay. A "conditional approval" description permits a company to release a drug to the market while providing additional time to collect efficacy data in the target species; a "designation" description allows a pharmaceutical company to apply for grants to offset the financial outlay needed to complete the work required to meet FDA approval; "indexing" applies to drugs that would be used so rarely that complete studies could not reasonably be conducted and does not cover drugs that would be used in food animal species. Additionally, companies may be eligible for reduced or eliminated user fees associated with NADA. The Office of the Minor Use/Minor Species (OMUMS) is responsible for the oversight of these procedures and communication with the Office of New Animal Drug Evaluation (ONADE).

National Research Support Project 7

National Research Support Projects (NRSP's) were established by the USDA to provide support to organizations conducting research in a specific area or discipline. The goal of these programs is to coordinate efforts and provide resources, information, materials, and facilities to accomplish research objectives in a defined discipline. The NRSP-7 Minor Use

Animal Drug Program identifies animal drug needs in minor species and coordinates data sharing and dissemination between animal industries, pharmaceutical companies, and research institutions with the ultimate goal of facilitating FDA drug approval for these underserved species or conditions. Currently 342 drug requests identified as valuable for use in minor species have been submitted to OMUMS for investigation, with over 100 of those drugs identified as urgently needed in minor species (United States Department of Agriculture National Research Support Project7, 2008). Prioritization of drug needs and indications with the greatest potential impact across the range of minor species is critically evaluated to ensure research aimed at meeting FDA requirements for drug approval addresses the needs of the stakeholders involved in these industries.

Macrolide antibiotics

Tulathromycin is a novel macrolide antimicrobial with a triamilide structure found to be effective against bacterial respiratory pathogens in cattle and swine (Benchaoui *et al*., 2004; Nowakowski *et al*., 2004). Since goats are susceptible to many of the same respiratory pathogens as cattle and the susceptibility of these agents to tulathromycin has been demonstrated in many *in vitro* and *in vivo* studies, tulathromycin was identified for investigation as a potentially suitable therapeutic agent against bacterial pneumonia in goats.

The macrolide class of antibiotics contain a 14-, 15-, or 16-membered macrocyclic lactone ring to which amino sugars are attached (Alvarez-Elcoro & Enzler, 1999; Retsemea & Fu, 2001). The unique structure of macrolides facilitates rapid dissemination from the central compartment (plasma) to lung tissues which make them useful in the treatment of bacterial pneumonia (Amsden, 2001; Williams & Sefton, 1993). Natural macrolides were

originally isolated from *Streptomyces;* first generation, semi-synthetic macrolides such as erythromycin were derived from these bacterial analogues (Alvarez-Elcoro & Enzler, 1999; Evans, 2005) . This class of antibiotic acts by binding to the 50S ribosomal subunit, preventing translocation of the growing peptide and blocking protein synthesis (Alvarez-Elcoro & Enzler, 1999; Benchaoui, *et al*., 2004; Evans, 2005; Retsemea & Fu, 2001; Williams & Sefton, 1993). Newer macrolides have been designed with modified configurations to enhance *in vitro* and *in vivo* antibacterial properties along with increasing bioavailability, lung tissue penetration, and extended tissue half-lives (Benchaoui, *et al*., 2004; Retsemea & Fu, 2001). The addition of a methyl group to the ring structure of azithromycin confers a two- to eight-fold increase in activity against *Haemophilus influenzae* over erythromycin (Retsemea & Fu, 2001).

Due to the unique pharmacokinetic (PK) and pharmacodynamic (PD) properties resulting in rapid dissemination from plasma and into tissues, macrolide behavior and potential efficacy cannot be fully evaluated with plasma concentration analysis that is used to assess other antimicrobials (Amsden, 2001; Benchaoui *et al*., 2004; Nightengale, 1997; Veber *et al*., 1993). Other PK/PD parameters that can be calculated from a time-concentration curve for a specific drug can be used to estimate total drug exposure and have been used to describe efficacy of macrolides and are shown in Figure 1.1. The time above minimum inhibitory concentration (T > MIC) for a specific pathogen correlates most directly with clinical efficacy in older macrolides (Nightengale, 1997; Veber *et al*., 1993). This parameter, however, is not valid when evaluating newer macrolides such as azithromycin and dirithromycin (Benchaoui *et al*., 2004). Tissue drug concentrations at the site of infection

may provide more relevant information to assess PK/PD activities of this drug class, particularly for novel compounds with enhanced lung persistence (Benchaoui *et al*., 2004; Evans, 2005). The newer macrolides achieve high concentrations in granulocytes, lymphocytes, and monocytes which will be directed toward sites of inflammation/infection and increase effective therapeutic drug concentrations in critical locations (Amsden, 2001). An alternative PK/PD parameter, the area under the time-concentration curve (AUC) compared to the minimum inhibitory concentration (MIC) of a specific pathogen, provides a much more valuable assessment for these drugs (Amsden, 2001). Frequently the AUC/MIC ratio is evaluated using both plasma and tissue analysis to assess potential macrolide efficacy.

Tulathromycin Properties

Tulathromycin is a novel macrolide that *in vivo* exists in a 9:1 ratio of 15-membered and 13-membered ring structures containing two attached sugar moieties (Evans, 2005; Nowakowski *et al*., 2004; Gáler *et al*., 2004). Both isoforms contain three amine groups at different locations on the molecule, placing tulathromycin in the triamilide group of macrolides (Evans, 2005). The presence of amine groups is a characteristic of macrolides, including erythromycin which contains one amine group and azithromycin which contains two; however, the tribasic structure provides unique attributes to this molecule (Evans, 2005). One amine group is contained on the azalide ring while the other two are attached to the sugar molecules in separate regions of the structure. Each amine may be neutral or positively charged so that each molecule can carry a charge of zero to 3+; all forms will exist in dynamic equilibrium depending on environmental pH (Evans, 2005; Nowakowski *et al*., 2004).

In addition to impacting enhanced tissue and cellular penetration characteristic of all macrolides, this novel structure conveys desirable anti-bacterial properties particularly against Gram negative respiratory bacteria. Cellular permeability is enhanced when molecules can exist in an un-ionized form (Goodman & Gilman, 2006). In its triply-charged form, tulathromycin is thought to displace Mg^{2+} ions from the cell wall of many Gram negative bacterial pathogens and damage cell wall integrity (Evans, 2005; Nowakowski *et al*., 2004). The molecules can then pass through the cell wall and into the periplasmic space, where equilibrium of the charged and uncharged molecules is re-established. Uncharged molecules then penetrate the inner cell membrane of these bacteria and gain access to cytoplasmic ribosomes (Benchaoui *et al*., 2004; Evans, 2005). As a macrolide, tulathromycin exerts its activity through binding to the 50S subunit of bacterial ribosomes and blocking peptidyl transferase which results in dissociation of transfer RNA (tRNA), cessation of peptide translocation, and blockage of protein synthesis (Benchaoui *et al*., 2004; Evans, 2005). Although this drug is classified as bacteriostatic, it can also exhibit bactericidal activity at higher concentrations (Benchaoui *et al*., 2004; Evans, 2005; Nowakowski *et al*., 2004).

As described earlier with other recently developed macrolides, standard pharmacodynamic assessment of antibiotic activity does not readily apply to tulathromycin (Benchaoui *et al*., 2004; Evans, 2005). In addition, due at least in part to its lipophilic properties, tulathromycin demonstrates better tissue penetration and longer half-lives than older macrolides (Benchaoui *et al*., 2004; Evans, 2005). This activity can provide unique therapeutic advantage in treating bacterial respiratory infections in livestock species.

Analysis of lung tissue homogenates provides an estimation of combined extracellular and intracellular drug concentrations but cannot differentiate between the two; however, in the case of macrolide antimicrobials, these values correlate well with magnitude of drug exposure at the site of infection (Benchaoui *et al*., 2004). This provides better data on potential *in vivo* efficacy than serum or plasma concentrations in this drug class (Benchaoui *et al*., 2004). Additionally, macrolides have properties that do not rely on drug concentration equilibrium between serum/plasma and tissue concentrations, such as permeation through nonporous membranes, pressure gradient bulk flow, and active transport that may account for macrolide behavior (Benchaoui *et al*., 2004).

Tulathromycin has been shown to accumulate in leukocytes (primarily macrophages and neutrophils) in greater amounts than older macrolides (Evans, 2005). In a study utilizing radio-labelled tulathromycin and erythromycin, tulathromycin demonstrated a 26-fold greater influx into and slower release from bovine neutrophils over erythromycin (Siegel *et al*., 2004). Neutrophil lysis that occurs at the site of infection provides for additional drug release in the area of greatest bacterial populations (Evans, 2005).

Pharmacokinetic and pharmacodynamic analysis

Pharmacokinetics (PK) is the study of drug behavior *in vivo*, and is based on drug concentrations in body spaces over time (Bauer, 2004; Gabrielsson & Weiner, 2000). Measured parameters are based both on the properties of the drug and on the biological system in which the drug is studied (Bauer, 2004; Rescigno, 2010). Pharmacokinetic behavior is broadly divided into absorption, distribution, metabolism/biotransformation, and excretion (Gabrielsson & Weiner, 2000; Goodman & Gilman, 2006). Absorption is

considered complete for drugs administered intravenously (IV) but must be determined for those administered by other routes (intramuscular, subcutaneous, oral). It determines the bioavailability (F), the percentage of drug that will reach the intended destination which is generally the blood stream. Distribution measures the amount of drug that is dispersed from the central compartment (blood) into intracellular and interstitial fluid. Highly vascular tissues have enhanced perfusion, facilitating drug exposure particularly by compounds with high lipid solubility. The purpose of drug metabolism generally is to increase the water solubility of the compound in preparation for excretion, which is largely accomplished via renal or enterohepatic action (Goodman & Gilman, 2006).

Physiochemical traits of a drug such as size, lipid solubility, degree of ionization, and protein binding properties determine how it will traverse cell membranes and arrive at a target location, how it will persist at that location, and how it will be removed from that location (Goodman & Gilman, 2006). The goal of clinical PK is to use calculated parameters of the drug's behavior in the body to maximize therapeutic benefits while minimizing toxic side effects (Bauer, 2004; Goodman & Gilman, 2006).

Pharmacokinetic analysis is used to estimate parameters that characterize the behavior of a drug and can be done experimentally or through the use of modeling (Bauer, 2004). Pharmacokinetic modeling attempts to match drug behavior with mathematical equations that can be used to predict drug activity in other populations, with the ultimate goal of maximizing therapeutic benefits while minimizing the chance of adverse effects from a given medication (Bauer, 2004). Pharmacokinetic models that describe the behaviour of one drug may also be applied to estimate behavior of compounds with similar structures or

physiochemical properties (Resigno, 2003). Optimal models are retrodictive, agreeing with data from the experiment on which they are based; are predictive, correctly estimating what will be seen in future experiments; and are understood, so that the model fits in logically to what has been seen in other experiments (Rescigno, 2003).

Non-compartmental PK models of experimental results consider the entire body as a single "vessel" into which the drug is administered and from which the drug is removed; analysis using this model is referred to as NCA (Bauer, 2004; Gabrielsson & Weiner, 2000; Goodman & Gilman, 2006; Rescigno, 2003). These models make the fewest assumptions about how the drug is distributed without the need to describe movement from one location to another (Gabrielsson & Weiner, 2000). Several fundamental parameters are critical to establishing valid models from which other parameters can be determined (Bauer, 2004; Gabrielsson & Weiner, 2000; Rescigno, 2003). Clearance (CL), the amount of drug removed per unit of time, is the most important parameter to determine since it dictates the dose and frequency of drug administration needed to reach a steady-state concentration (Bauer, 2004; Goodman & Gilman, 2006). This parameter indicates how efficiently the body removes the specific drug and provides information on the balance needed between administration and elimination to keep drug levels within the therapeutic index. CL encompasses perfusion, diffusion, filtration, metabolism, and transport processes for the specific drug (Gabrielsson & Weiner, 2000). The volume of distribution (Vd) dictates how broadly the drug mobilizes throughout the body and is proportional to the amount of drug in the body; Vd is generally small if most of the drug is retained in the blood stream and large if the drug disseminates to tissues (Bauer, 2004; Gabrielsson & Weiner, 2000; Goodman &; Gilman, 2006). When the

route of administration is anything other than IV, bioavailability (F) will have an influence on these parameters; consequently, they become apparent clearance (CL/F) and apparent volume of distribution (Vd/F).

Other pharmacokinetic parameters are measured or calculated from these primary factors. Graphical representations of drug concentrations over time are used to calculate the area under the time-concentration curve (AUC), which gives an estimation of total drug exposure (Bauer, 2004; Gabrielsson & Weiner, 2000). NCA calculation of this parameter utilizes a sum of trapezoidal areas or non-linear regression; consequently, the more frequently measurements of drug concentration are collected, the smaller the magnitude of error in the calculation (Gabrielsson & Weiner, 2000; Rescigno, 2010). Half-life ($t_{½}$) is the time needed to remove half of the drug from the compartment under study (usually plasma but can also be applied to a specific tissue), and is dependent on both CL and Vd (Bauer, 2004; Gabrielsson & Weiner, 2000; Goodman & Gilman, 2006). The elimination rate of a drug is estimated by the determining the terminal slope (λ_z) of the time-concentration curve; the maximum drug concentration (C_{max}) and time to reach maximum drug concentration (T_{max}) are derived directly from the time-drug concentration plot (Gabrielsson & Weiner, 2000; Goodman & Gilman, 2006; Rescigno, 2003).

Compartmental analysis uses kinetic models to predict the time-concentration plot and considers drug entry and elimination from various segments of the body, such as blood, fat, or central nervous system (Gabrielsson& Weiner, 2000; Goodman & Gilman, 2006; Rescigno, 2010). Experimental results of drug behavior may not agree with non-compartmental models and more complex analysis that accounts for distribution and

redistribution of drugs from the central (blood stream) and peripheral (tissue) compartments may be required (Gabrielsson & Weiner, 2000). Additional parameters are utilized to describe how the drug moves through the body. Elimination is divided into two different phases described by the initial and terminal slopes and may more accurately describe the drug concentration at a particular time than NCA (Gabrielsson & Weiner, 2000; Rescigno, 2003). Model building is multifaceted utilizing multiple exponential terms and complex calculations which are made possible through the use of specialized computer programs. Once a model is complete, it is evaluated for precision and agreement with experimental results collected from additional studies (Bauer, 2004; Rescigno, 2003).

Information about pharmacokinetic parameters such as clearance and effective dose can be used to predict drug behavior in another species or with an alternative route of administration (Gabrielsson & Weiner, 2000). Allometric scaling has been used to extrapolate drug behavior in different species, utilizing a PK assessment of a drug in one species and physiologic properties of body size and metabolism in the alternate species to estimate drug behavior in the alternate species (Drew *et al*., 2004). More recently, physiologically-based pharmacokinetic (PBPK) models have been developed to more accurately evaluate drug behavior *in vivo*. These highly complex models involve multi-compartment analysis and can be used to predict drug disposition into and out of individual tissues (Gabrielsson & Weiner, 2000). PBPK models permit extrapolation of drug effects (both therapeutic and toxicological) between species and can be used to perform risk assessments from drug exposure to alternate species (Sweeney *et al*., 2009). Additionally,

they can be used to evaluate specific tissues such as skin or nasal mucosa that might not be easily incorporated into less complex models (Norman *et al*., 2010; Sweeney, 2009).

Pharmacodynamics (PD) is the study of the relationship between drug exposure, mechanism of action, and the biological effects of the drug on the host or in the case of an antibiotic, on the bacterial target (Gabrielsson & Weiner, 2000; Rescigno, 2010). While PK evaluates the concentration of a drug over time, PD focuses on the effects of the drug over a range of concentrations. Parameters used to evaluate PD effects of a drug include the minimum inhibitory concentration (MIC) against a specific bacterium *in vitro*, post-antibiotic effects (PAE), and kinetics of bacterial killing (Godinho *et al*., 2005; Wise, 2001). Drugs that demonstrate PAE maintain antibacterial activity for a period of time beyond when the drug concentration falls below the MIC for a specific pathogen (Wise, 2001). The relationship between drug concentration and effect is complex in biological systems; however, controlled *in vitro* PD experiments contribute to the development of mathematical descriptions of drug activity (Rescigno, 2003).

The two model descriptors can be combined to provide PK/PD assessment of a drug and its intended activity. Antibiotics are traditionally classified into one of three categories, time-dependent, concentration-dependent, and time-dependent with a post-antibiotic effect, based on PK/PD activity (McKellar *et al*., 2004). The activity of time-dependent antimicrobials relies on the length of time that drug levels are at or above the MIC (T>MIC) of the specific pathogen being treated; the longer drug levels persist above the MIC, the more effective the antibiotic treatment. Common time-dependent antibiotics include penicillins, cephalosporins, and older macrolides (Van Bambeke & Tulkens, 2001). Concentration-

dependent antimicrobials rely on the maximum level of antimicrobial above the MIC (C_{max}/MIC) of the pathogen being treated and frequently demonstrate PAE; effective treatment is based upon maximizing plasma or tissue concentrations of the drug and benefits from the suppression of bacterial growth that continues for a period of time even after drug levels fall below the MIC. Aminoglycosides, tetracyclines, and fluoroquinolones are members of this category (Van Bambeke & Tulkens, 2001). The third category of antibiotics, which includes streptogramins, ketolides, and newer macrolides, has characteristics of both, with time-dependent killing mechanisms and PAE. PAE is based on the concentration of drug, the time that bacteria are in contact with the drug, and/ or its mechanism of action (Van Bambeke & Tulkens, 2001). Total drug exposure is evaluated by the area under the time-concentration curve (AUC) and represents a mathematical summation of drug concentrations in the compartment being assessed (Van Bambeke & Tulkens, 2001; Gabrielsson & Weiner, 2000). The AUC/MIC ratio is frequently used to determine drug efficacy of this group (Van Bambeke & Tulkens, 2001; Li & Zhu, 2002). PAE may be due to the effects of altered DNA or protein synthesis (Li & Zhu, 2002).

Effects of drug exposure on a bacterial species are important aspects of determining potential drug efficacy. Minimum inhibitory concentration (MIC) determination involves incubating a bacterial suspension with a range of doubling antibiotic concentrations for a specified time and examining them for growth inhibition; MIC's are considered the "gold standard" of susceptibility testing (Andrews, 2001; Catry *et al*., 2007). The MIC is defined as the lowest concentration of antimicrobial that suppresses bacterial growth in a defined incubation period (Andrews, 2001). The Clinical and Laboratory Standards Institute

(formerly the National Committee for Clinical Laboratory Standards) establishes interpretive criteria for "susceptible," "intermediate," or "resistant" determinations between the bacterial isolate and the drug (Clinical and Laboratory Standards Institute, 2008).

Mean bactericidal concentrations (MBC) can be identified by sub-culturing the bacterial-antibiotic suspension used in MIC testing onto antibiotic-free media to determine what concentration of antibiotic is required for complete bacterial killing (Andrews, 2001). Bactericidal antibiotics have MBC that are very close to MIC values since their mode of action results in irreversible damage to the bacterium; bacteriostatic drugs have MBC that are much greater than MIC (Andrews, 2001). Kill-kinetic assays are an established method to determine the degree of bacterial killing (Firsov *et al*., 1997; Stratton *et al*., 1987). In this testing method, a growing bacterial suspension is incubated with a known concentration of antibiotic and serially tested to establish the drug concentration and time needed to achieve a defined level of bacterial death (Firsov *et al*., 1997; Stratton *et al*., 1987). The most common chosen levels of killing are MBC_{90} (90 % killed), MBC_{95} (95% killed), and MBC_{99} (99% killed).

Drug elimination is also a critical component of *in vitro* behavior, particularly in food animal species from which products are expected to enter the human food chain. Tulathromycin is manufactured as a single isomer or parent compound, designated CP-472,295(e). This molecule equilibrates in solution into in 9:1 mixture of two tulathromycin isoforms, designated CP-472,295 and 547,272, respectively (Gáler *et al*., 2004). The common fragment or marker residue for these two different isoforms is CP-60,300 (Gáler *et al*., 2004). Two different methods for tulathromycin detection have been described for

different biological matricies. Since tissue residues in food animal species are used to establish adequate withdrawal periods after drug administration, detection protocols that identify drug compounds in these tissues must receive regulatory approval. Frequently these methods are designed to meet stringent guidelines that maximize detection of the drug in any form. Tissue residue studies are therefore conducted utilizing a FDA-approved methodology detecting the common fragment CP-60,300.

Determination of tissue concentrations is a necessary method of evaluating certain drugs for efficacy, particularly macrolide antimicrobials. An accurate, precise, and robust detection method is needed to characterize a drug's distribution in the animal and its relationship to efficacy (Gáler *et al*., 2004). A detection method that focuses on the most active structure of the drug being evaluated provides more information on the potential therapeutic potential of the specific medication than overall tissue concentrations. The method described by Gáler *et al* (2004) focuses on the evaluation of the parent compound of tulathromycin (CP-472,295[e]) in lung tissues and plasma since these matricies are critical to determining potential drug efficacy in the tissue(s) being targeted for therapy.

Bacterial pneumonia

Bacterial respiratory disease is a common health concern in small ruminants with potential life-threatening consequences (Ackermann & Brogden, 2000; Berge *et al*., 2006; Brogden *et al*., 1998; Martin, 2010; Washburn *et al*., 2007; Yener *et al*., 2009). The most frequently implicated bacterial agents in this disease complex are *Mannheimia haemolytica, Pasteurella multocida,* and *Bibersteinia (Pasteurella) trehalosi,* often complicated by *Mycoplasma* species (Ackermann & Brogden, 2000; Alley *et al*., 1999; Berge *et al*., 2006;

Brogden *et al*., 1998; Cutlip *et al*., 1998; Rahman & Singh, 1990; Shayegh *et al*., 2009; Washburn *et al*., 2007; Yener *et al*., 2009; Zamri-Saad & Mera, 2001). *B. trehalosi*, previously classified as *M. haemolytica* type T but now identified as a distinct species, utilizes many of the same virulence mechanisms as *M. haemolytica*. An antimicrobial shown to be effective against these pathogens would be highly useful in caprines.

Pathogenesis of bacterial pneumonia in goats is similar to that seen in other livestock species. Upper respiratory viruses such Parainfluenza 3 (PI-3), adenovirus type 6, respiratory syncytial virus (RSV), bovine adenovirus type 2, ovine adenovirus types 1, 2, and 5, caprine arthritis encephalitis virus, and reovirus type 1, as well as *Mycoplasma* bacteria have all been identified as agents capable of causing primary pneumonia in small ruminants (Brogden *et al*., 1998; Murphy *et al*., 2010; Purdy *et al*., 2003; Yener *et al*., 2005). More importantly, these agents damage host defenses and predispose the lung to bacterial entry into lungs. Stress associated with weather extremes, weaning, transport, and poor nutrition have all been implicated as predisposing factors associated with the development of pneumonia (Brogden *et al*., 1998; Purdy *et al*., 2003; Shafarin *et al*., 2009). *M. haemolytica*, *P. multocida*, and *B. trehalosi* are all commensals of the upper respiratory tract in ruminants that contribute to severe pneumonia upon reaching the lungs (Ackermann & Brogden, 2000; Autio *et al*., 2007; Confer, 2009; Rice *et al*., 2007; Ward *et al*., 2002; Whiteley *et al*., 1992; Yener *et al*., 2009). These commensal agents have been recovered from nasal and pharyngeal samples collected from goats, sheep, and cattle (Shiferaw *et al*., 2006; Tomassini *et al*., 2009). Any conditions resulting in suppressed immunity can predispose the animal to entry of these agents into deeper lung tissue and the development of pneumonic pasteurellosis (Brogden *et al*., 1998;

Cutlip *et al*., 1996; Dassanayake *et al*., 2010; Ewers *et al*., 2006). Additionally, neutralizing antibodies to bovine respiratory viruses have been documented in small ruminants, with Bovine Herpesvirus-1 (BHV-1) and PI-3 showing the highest seroprevalence rates in goats (Yeçilbag & Güngör, 2008). The evidence of a serologic immune response in these animals indicates that these viruses are capable of contributing to upper respiratory mucosal damage and thus secondary bacterial entry into the lungs. Co-localization of PI-3 and *M. haemolytica* identified by immunohistochemical (IHC) staining in association with fibrinous bronchopneumonia demonstrated by Yener *et al* (2005) may indicate that PI-3 has an important role in caprine pneumonia. Goats are also frequently used as models for studying respiratory disease in cattle due to the similarity of these two species in susceptibility to inciting causes, bacterial pathogens, and mechanisms of pathology (Purdy *et al*., 1998).

The pathogenesis of the common respiratory diseases in cattle has been well described (Ackermann & Brogden, 2000; Angen *et al*., 2009; Autio *et al*., 2007; Confer, 2009); Czuprynski *et al*., 2004; Haines *et al*., 2004; Mosier, 1997; Rice *et al*., 2007) and parallel disease development has been observed in goats. Infection of upper and lower respiratory tissues by agents such as Bovine Herpes Virus 1 (BHV1), Bovine Viral Diarrhea Virus (BVDV), Parainfluenza 3 virus (PI3), and Bovine Respiratory Syncytial Virus (BRSV) result in damage to upper respiratory epithelium and local mucosal immunosuppression, facilitating lung infection by bacterial agents (Angen *et al*., 1999; Autio *et al*., 2007; Confer (2009); Czuprynski *et al*., 2004; Ewers *et al*., 2004; Fulton *et al*., 2009; Gagea *et al*., 2006; Hodgson *et al*., 2005; Rice *et al*., 2007). *Mycoplasma bovis* has been shown to both predispose to and benefit from the presence of other respiratory pathogens in contributing to pulmonary lesions

(Nicholas & Ayling, 2003; Razin *et al*., 1998; Vanden Bush & Rosenbusch, 2003; Vanden Bush & Rosenbusch, 2004). As is seen in goats, stress from weaning, weather extremes, transport, and comingling can contribute to depressed host immunity in cattle, predisposing to establishment of bacterial lung infection (Hodgson *et al*., 1996; Rice *et al*., 2007; Straus *et al*., 1998).

Pneumonic agents are often found in combination within diseased tissues, providing evidence of the opportunistic activity of normal upper respiratory flora (Srikumaran *et al*., 2007). Infections by upper respiratory pathogens incite the release of pro-inflammatory cytokines IL-1, IL-8, TNF-α, and recruitment of phagocytic cells (Hodgson *et al*., 1996). Damage to upper respiratory defense mechanisms by viral agents includes breakdown of β-defensins, anionic peptides and mucous secretions; decreased clearance by the mucociliary escalator; and aerosolization of pharyngeal microbiota; the result is bacterial aspiration and establishment of pulmonary infections (Ackermann & Brogden, 2000; Rice *et al*., 2007).

Characteristic lesions of pneumonic pasteurellosis are similar in goats, cattle, and sheep and include an acute fibrinonecrotic pneumonia associated with infiltrates of neutrophils, fibrin, and proteinaceous fluid (Ackermann & Brogden, 2000; Fett *et al*., 2008; Purdy *et al*., 2003; Yener *et al*., 2009; Zamri-Saad & Mera, 2001). Shafarin *et al*. (2009) demonstrated these gross and histopathologic lesions in goats challenged with *P. multocida*. Pneumonic goat lungs that were examined by immunohistochemistry (IHC) specific for *M. haemolytica* showed strongest staining in epithelial cells of small bronchioles and pneumocytes as well as in degenerating neutrophils and macrophage-like cells particularly in areas of necrosis (Yener *et al*., 2009). Alveolar macrophages from goats challenged with *M. haemolytica*

showed depressed phagocytic activity compared with macrophages from goats challenged with other respiratory agents (Zamri-Saad & Mera, 2001). The severe tissue damage seen with these bacterial pathogens demonstrates the need for effective antimicrobial therapy.

Similarities and differences between goat and cattle production in the U.S. highlight diverse challenges associated with the epidemiology of bacterial pneumonia in goats. Goats and cattle are subjected to many of the same stresses associated with increased susceptibility to clinical pneumonia, such as weaning, weather extremes, and nutritional deficiencies (Ackermann & Brogden, 2000; Brogden *et al*., 1998; Czuprynski *et al*., 2009; Hodgson *et al*., 1996; Murphy *et al*., 2010; Purdy *et al*., 2003; Rice *et al*., 2007; Straus *et al*., 1998; Yener *et al*., 2005). Susceptibility to upper respiratory agents, particularly BHV-1 and PI-3, has also been demonstrated in both species. The similarities in resident pharyngeal flora facilitate exposure to opportunistic lung pathogens in both bovine and caprine species. Several management practices associated with cattle production increase the risks for this disease complex, including co-mingling of animals from different sources at barns and auctions; intensive management associated with finishing in feedlots; and long-distance shipping to these feeding operations. Most market goat feeding systems rely on pasture-based, extensive management, which decreases the risk for the spread of infectious agents associated with close contact in small pens. Extensive management, however, does present different challenges such as less frequent monitoring for signs of disease development and the nutritional stresses associated with marginal forage systems. In addition, a plethora of vaccines directed against upper and lower respiratory disease agents are labeled for use in cattle, and pre-conditioning programs are more widely implemented to bolster immunity

prior to contact with stressful events. No such vaccines are available for use in goats and pre-conditioning, which is extremely difficult to accomplish in extensively managed operations, is a rare occurrence in caprines.

Bacterial pneumonia agents

M. haemolytica is a Gram negative coccobacillus in the Pasteurellaceae family. It was originally classified into 16 serotypes based on surface antigens that reacted in an agglutination reaction (Biberstein, 1978). A novel serotype, A17, was identified in sheep in 1995 (Rice *et al.*, 2007). Further characterization divided the bacteria into 13 "A" biotypes, which ferment arabinose, and 4 "T" biotypes, which ferment trehalose (Blackhall *et al*., 2007; Lo & Shewen, 1991). Sequencing of 16S rRNA determined that these biotypes were actually distinct species; 12 of the A biotypes are currently assigned to *M. haemolytica* and one to *M. glucosida* while the four T biotypes are designated as *B. (Pasteurella) trehalosi* (Angen *et al*., 1999; Rice *et al*., 2007; Sneath & Stevens, 1990).

M. haemolytica is considered the major pathogen in ruminant respiratory disease (Frank & Briggs, 1992; Rice *et al.*, 2008). Serotypes A1, A2, and more recently A6 are the most commonly recovered serotypes colonizing the nasopharynx of cattle, sheep, and goats, with other serotypes being identified infrequently (Fett *et al*., 2008; Frank & Briggs, 1992; Frank & Smith, 1983; Jaramillo-Arango *et al*., 2008; Rice *et al.*, 2008). Clinical pneumonia is most frequently caused by serotype A1 in cattle while in small ruminants A2 is the main identified serotype (Dassanayake *et al*., 2009; Highlander, 2001; Odugbo *et al*., 2004; Rice *et al.*, 2008). A review of isolates from clinical lesions in goats found that serotype A2 represented 92% of all *M. haemolytica* isolates identified (Fodor *et al*., 1999). *M. haemolytica* and *B.*

trehalosi have been isolated from pharyngeal swabs of healthy goats, indicating that opportunistic entry into lung tissue is possible in goats (Ward *et al*., 2002).

M. haemolytica causes severe fibrinonecrotic pneumonia in ruminants but not in other species (Ackermann & Brogden, 2000; Deshpande *et al*., 2002; Zecchinon *et al*., 2004). The two main virulence factors associated with this pathogen are lipopolysaccharide (LPS), which is common in infections with all Gram negative bacteria, and a secreted leukotoxin (Lkt) that primarily targets ruminant leukocytes (Atapattu & Czuprynski, 2007; Baluyut *et al*., 1981; Clinkenbeard *et al*., 1989; Dassanayake *et al*., 2007a; Dassanayake *et al*., 2007b; McClenahan *et al*., 2008; Sacco *et al*., 2006). LPS can produce cytotoxicicity and apoptosis in bovine endothelial cells (McClenahan *et al*., 2008; Paulsen *et al*., 1989). Additionally, LPS binds to and stabilizes Lkt, preventing its degradation and augmenting its activity (Jeyaseelan *et al*., 2002). Lkt, a calcium-dependent cytotoxin, is a member of the RTX family of toxins produced by many Gram negative bacteria (Atapattu & Czuprynski, 2007; Baluyut *et al*., 1981; Clinkenbeard *et al*., 1989; Dassanayake *et al*., 2007a; Dassanayake *et al*., 2008; Deshpande *et al*., 2002; Zecchinon *et al*., 2004; Zecchinon *et al*., 2005). Lkt activity is responsible for much of the gross and histopathologic tissue damage seen with *M. haemolytica* and *B. trehalosi* infections (Clinkenbeard *et al*., 1989; Dassanayake *et al*., 2007b; Jeyaseelan *et al*., 2002; Leite *et al*., 2005; Zecchinon *et al*., 2005). Species susceptibility to this bacterial pathogen may be characterized by the ability of Lkt to activate leukocytes in cattle, sheep, and goats but not in other species (Dassanayake, *et al*., 2007a ; Dassanayake *et al*., 2007b ; Fett *et al*., 2008 ; Lawrence *et al*., 2007 ; Lawrence *et al*., 2008 ; Zecchinon *et al*., 2005).

As more microbiologists have differentiated *M. haemolytica* from *B. trehalosi* through the use of specific biochemical or molecular testing, the importance of *B. trehalosi* as a pathogen in sheep and goats is increasingly recognized. Leukotoxin-producing strains of *B. trehalosi* have been associated with clinical pneumonia in sheep (Dassanayake *et al*., 2010). Dassanayake *et al*. (2010) identified that *B. trehalosi* has a shorter doubling time and reaches a 3-log higher cell density after 5 hours of incubation *in vitro* than *M. haemolytica*. *B. trehalosi* is able to inhibit growth of *M. haemolytica* in an as yet uncharacterized, contact-dependent mechanism, indicating that it may have greater pathogenic potential than previously documented (Dassanayake *et al*., 2010).

While the pathogenic processes associated with *M. haemolytica* appear to be limited to ruminants, clinical conditions related to *P. multocida* infections have been identified in a range of species, including swine, dogs, rabbits, and chickens (Davies *et al*., 2003; Ogunnariwo & Schryvers, 2001; Weber *et al*., 2004). This pathogen is most frequently identified with primary respiratory disease in goats, although rarely hemorrhagic septicemia has been reported (Ewers *et al*., 2004; Shafarin *et al*., 2009).

P. multocida is a highly heterogeneous species with four recognized subspecies: multocida, gallicida, septica, and tigris (Shayegh *et al*., 2009). Additionally, there are five identified "capsular types" (A, B, D, E, and F) with differing antigenic specificities (Dabo *et al.*, 2007; Shayegh *et al*., 2009). Type A is the most common capsule types recovered from goat lesions although occasionally Type D has been reported (Ewers *et al*., 2006; Shayegh *et al*., 2009). Strains of *P. multocida* that are positive for the *tox*A gene express a dermonecrotic toxin that activates intracellular signalling cascades, producing severe tissue

damage, endothelial leakage, and cell lysis (Ewers *et al*., 2006; Shayegh *et al*., 2009; Wilson & Ho, 2004). Additional virulence factors include two transferring binding proteins encoded by *tbp*A and *tbp*B; a filamentous hemagglutinin encoded by *pfh*A; and a tight-adherence factor that thwarts host immune response and is up-regulated in the presence of commonly prescribed antibiotics (Melnikow *et al*., 2008; Shafarin *et al*., 2007; Shayegh *et al*., 2009). Lesions associated with *P. multocida* pneumonia include acute to subacute bronchopneumonia with occasional pleuritis (Dabo *et al*., 2007; Shafarin *et al*., 2007).

Various species of *Mycoplasma* can cause and contribute to respiratory disease in goats, including *M. ovipneumonia, M. capricolum, M. mycoides* Large Colony, *M. mycoidies ssp. capri, M. putrifaciens*, and *M. arginini* (Orós *et al*., 1997; Rodríguez *et al*., 1996). *M. capricolum ssp. capripneumoniae* causes caprine progressive pleuropneumonia and can result in widespread outbreaks with severe economic consequences (Chazel *et al*., 2010). Pneumonia and pleuritis have been associated with *M. ovipneumoniae* in goats; however, this agent has also been isolated from the upper respiratory tract of clinically normal goats, indicating that it may act as an opportunist to gain entry into the lungs (Goltz *et al*., 1986). *Mycoplasma* in sheep and goats can be seen in conjunction with other respiratory bacteria, particularly *M. haemolytica* and *P. multocida* (Gonçalves *et al*., 2010; Oros *et al*., 1997). In a challenge study in bighorn sheep involving a combination of *M. ovipneumoniae* and *M. haemolytica*, three of four dual-challenged animals developed fatal pneumonia 1-5 days post-challenge. The remaining animal died of pneumonia before challenge with *M. haemolytica* but its lungs were culture-positive for both *M. ovipneumoniae* and *M. haemolytica*; an identical strain of *M. haemolytica* was identified from the nasopharynx, signifying that

resident or introduced *M. haemolytica* can benefit from the presence of *M. ovipneumoniae* in establishing respiratory infections (Dassanayake, 2010).

Characteristics of this genus include small size, lack of a cell wall, and a highly variable surface protein structure that facilitates evasion of host immune responses and persistence in affected animals (Caswell & Archambault, 2007; Razin *et al*, 1998; Vanden Bush & Rosenbusch, 2003). Genomes encode for a family of highly antigenic variable surface proteins (VSP's); these genes undergo high rates of recombination which results in altered antigenic presentation, immune evasion, and persistence in the host (Caswell & Archambault, 2007; Razin *et al*., 1998; Vanden Bush & Rosenbusch, 2004). Pathologic lesions include subacute suppurative bronchiolitis, alveolitis, pleuritis, bronchopneumonia, fibrinonecrotic pleuropneumonia, and dilation of interlobular septa (Goltz *et al*., 1986; Rodríguez *et al*., 1996).

Meeting FDA Requirements

Safety

The purpose of drug safety studies is to determine if any undesirable side effects if any can be attributed to administration of the drug to the target species. The therapeutic index (TI) of a compound is defined as the ratio of the lethal dose in 50% of the population compared to the minimum effective dose in 50% of the population and provides an indication of the relative safety of that drug (Burns, 1999). Drugs with larger TI have a wider margin of safety since the dose associated with death is much greater than the dose needed to achieve effective therapy (Burns, 1999). To assess target animal safety, the effective dose and progressively higher doses are administered for an extended duration to elicit detrimental

effects in test subjects. A variety of assessments are used to evaluate these effects including physical, physiologic, and tissue parameters. Additionally, the target population is selected to represent the animals most likely to demonstrate harmful outcomes if any occur.

One study has been reported that assesses the safety of tulathromycin administration in goats (Washburn *et al*., 2007). Adult goats in this study were injected with 25 mg/kg which is ten times the label dose (2.5 mg/kg) for cattle and swine and observed for signs of adverse reactions. Observations on physical condition (survival, general attitude, fecal consistency, appetite, and injection site lesions); clinical pathology parameters (complete blood count, serum chemistry, and urinalysis); and genotoxicity assessment using flow cytometry-measured DNA content as a measure of chromosomal abnormalities associated with tulathromycin exposure were conducted for seven days following drug administration. The only parameters found to be significantly different between treatment and control animals were heart rate ($P = 0.04$) and creatine kinase ($P = 0.001$). Studies designed to meet FDA standards generally require extensive outcome assessments including gross and histopathologic tissue evaluations in addition to ante mortem appraisals described in this study.

Human Food Safety

A highly sensitive and specific detection method is needed to ensure accurate determinations of drug concentrations in plasma and tissues (Gáler *et al*., 2004). To meet FDA requirements for drug residue analysis, a method must maximize sensitivity and accuracy in detecting the compound. The method that identifies a common fragment of the various forms of tulathromycin (CP-60,300) was designed by Pfizer Animal Health and

approved as the residue detection method by the FDA CVM. The inclusion of samples that have been spiked with known tulathromycin concentrations along with analysis of incurred tissues from treated animals helps to minimize any analytical bias in that may occur from using only one sample type (Gáler *et al*., 2004). To utilize this procedure on goat tissues, the method must be validated using goat tissue samples spiked with known concentrations of tulathromycin; incurred tissues from tulathromycin-treated goats; and tissues from untreated goats. Validation involves assessment of several parameters including specificity, linearity, recovery, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and stability of the extracts. Furthermore, the method needs to be bridged to at least one of the species evaluated in the FDA-approved method.

Once the method is validated, it can be used to determine concentrations of the common fragment or marker residue CP-60,300 in tissues from market age animals that would be most likely to enter the human food supply. Animals are injected with the proposed label dose and tissues are collected and analyzed for tulathromycin content at various time points following injection. This data can then be used by the FDA to establish an adequate withdrawal period in the target species.

Efficacy

In order to meet the standards that FDA requires for antimicrobial approval, the drug must be shown to be effective in the species under study. This can be accomplished by more than one method. Ideally one would utilize the compound in natural outbreaks of bacterial disease since challenge studies with pathogenic organisms may not completely mimic what is seen in field conditions. Due to the intensive nature of cattle and swine production in the

U.S., efficacy studies in these species could be conducted under Good Clinical Practice (GCP) conditions utilizing tulathromycin compared to an alternative antimicrobial regimen to treat naturally occurring bacterial pneumonia. Many studies have been published that evaluate the efficacy of tulathromycin in the treatment of cattle and swine pneumonia (Booker *et al*., 2007; Evans, 2005; Godinho *et al*., 2005; Hart *et al*., 2006; Kilgore *et al*., 2005; Nickell *et al*., 2008; Perrett *et al*., 2008; Schunicht *et al*., 2007; Van Donkersgoed *et al*., 2008; Wellman & O'Connor, 2007).

Goat production is more extensive than that used in cattle and swine, with very few operations that raise large numbers of goats in small areas (Glimp, 1995). Requirements for adherence to GCP standards and treatment of naturally occurring pneumonia would be difficult if not impossible to accomplish in a defined time period in these production systems; therefore, an alternative method based on pharmacokinetics in the target species along with assessments of bacterial susceptibility was selected to assess tulathromycin efficacy in caprines.

To assess efficacy in cattle and swine, PK studies of tulathromycin behavior were conducted in addition to studies utilizing tulathromycin treatment in cases of naturally occurring clinical disease (Benchaoui *et al*., 2004; Nowakowski *et al*., 2004). A PK study conducted in goats to evaluate drug absorption and distribution as well as to perform PK analysis would provide valuable data that may help to establish efficacy. Additionally, lung tissue analysis for the parent compound of tulathromycin (CP-472,295[e]) can further define lung concentrations of this drug. While examination of lung tissue homogenates is not a definitive method for detecting drug concentrations of extra-cellular fluid, this analysis does

provide insight into the PK/PD behavior of drugs for which serum and plasma data are not useful (Benchaoui *et al*., 2004). In the case of long acting macrolides such as tulathromycin, PK/PD assessments based on lung tissue concentrations correlate better with total drug exposure and clinical efficacy than PK/PD assessments made on blood (Benchaoui *et al*., 2004). Results of PK behavior of tulathromycin in goats could then be compared to that found in cattle and swine. Profiles obtained from the PK study could also permit calculation of the AUC, an indication of total drug exposure, from both plasma and tissue compartments.

In vitro investigations into the effects of tulathromycin on respiratory bacterial pathogens can indicate the potential efficacy of this drug against caprine respiratory pathogens. Although goats and cattle share susceptibility to many of the same bacterial agents, differences in serotype, capsular type, and species (in the case of *Mycoplasma*) of recovered bacteria have been identified. Utilizing a collection of bacterial isolates that have been cultured from lung lesions in goats that have succumbed to clinical pneumonia would be a more effective way to assess tulathromycin efficacy against these pathogens. Evaluation of MIC, MBC, and killing ability through kinetic-kill assays can provide valuable insight into the true antibacterial activity of tulathromycin. Kinetic assays in particular can demonstrate bactericidal activities of the drug being evaluated (Dorfman, 2008). Employing the MIC values determined in these evaluations in the calculation of the AUC/MIC ratio will provide an effective means of assessing tulathromycin efficacy that has been shown to be useful with other new macrolides (Amsden, 2001).

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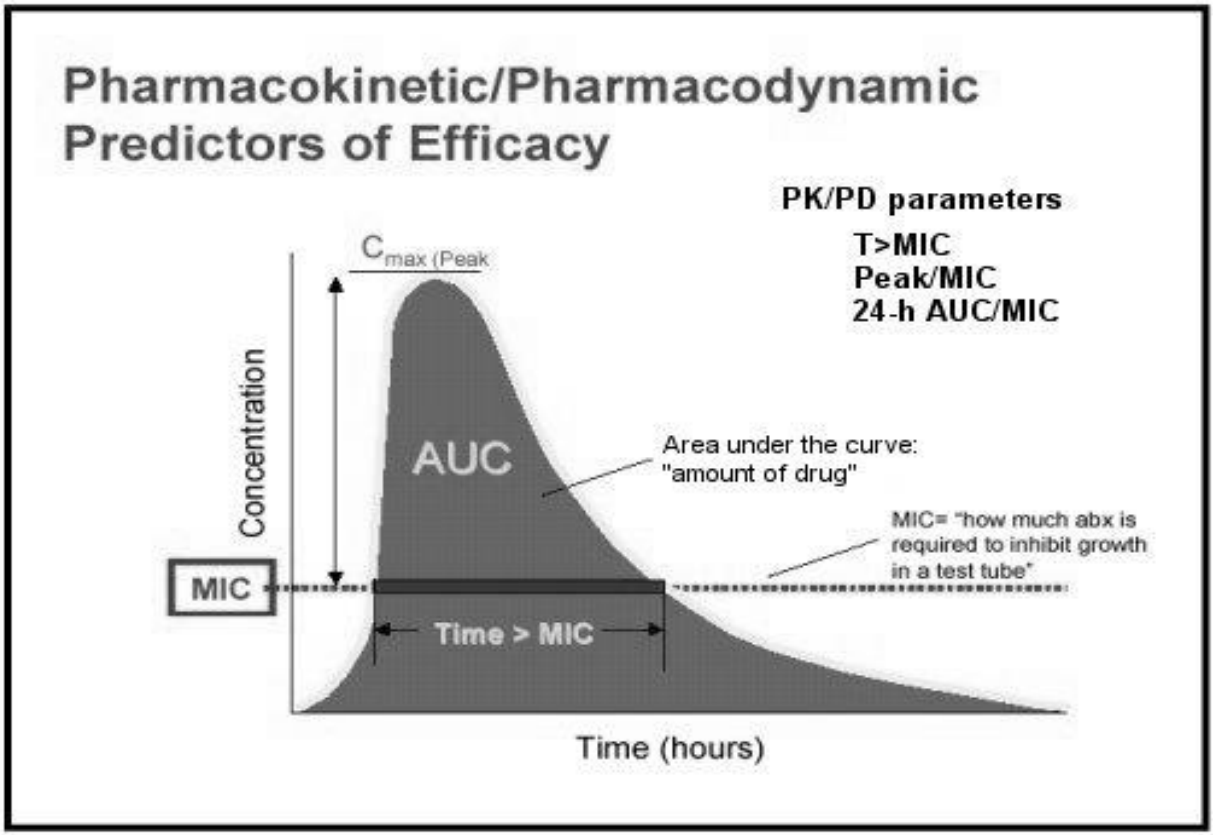

Figure 1.1 Pharmacokinetic/pharmacodynamic parameters used to predict efficacy of antimicrobials against specific bacterial pathogens. From "PK/PD approach to antibiotic therapy review", Rxkinetics software (www.rxkinetics.com/antibiotic_pk_pd.html, 8-16-10)

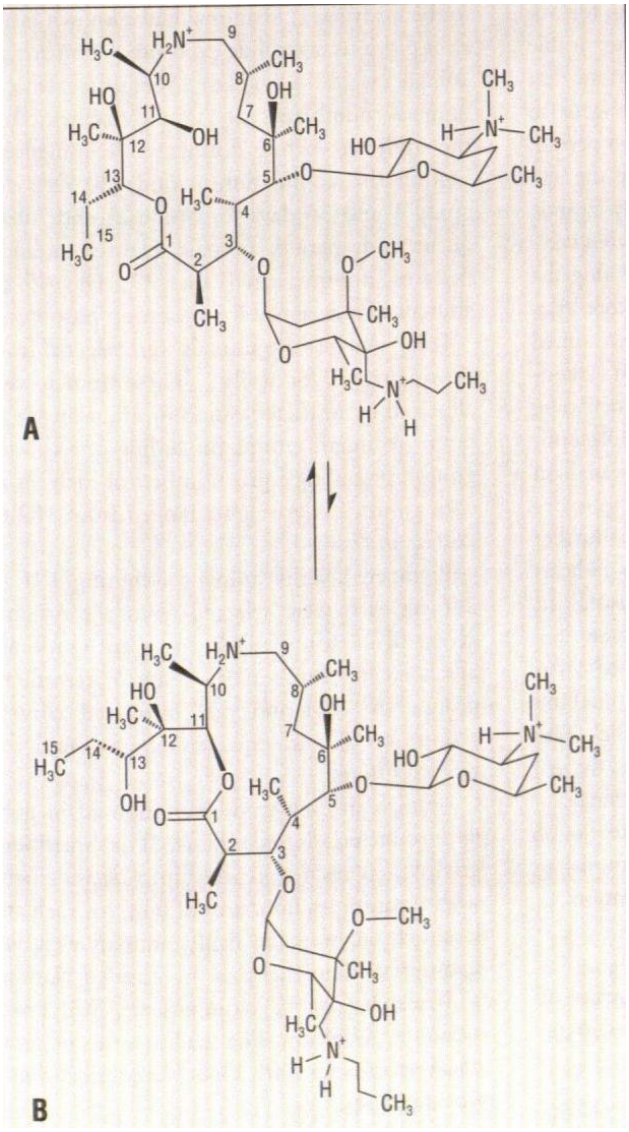

Figure .2. Structure of the two isomers of tulathromycin that exist in a 9:1 (A:B) ratio under physiologic conditions. From Evans, N.A. (2005) Tulathromycin: an overview of a new triamilide antibiotic for livestock respiratory disease. *Veterinary Therapeutics*, 6, 83-95.

CHAPTER 2. SAFETY EVALUATION OF TULATHROMYCIN USE IN THE CAPRINE SPECIES

Adapted from the manuscript published in the *Journal of Veterinary Pharmacology and Therapeutics*

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Abstract

Antimicrobials available to treat bacterial respiratory disease in goats are extremely limited. Currently only cephalosporins have been approved for use in this species. Concerns over antimicrobial resistance in human bacterial isolates have led to proposed restrictions on the use of this drug in food producing animals. Tulathromycin is a triamilide macrolide antibiotic labelled for use against respiratory pathogens in cattle and swine. The present study was conducted to evaluate the safety of tulathromycin in caprines using the youngest goats likely to be treated for respiratory disease. Twelve male and 12 female, one week old, mixed-breed goats were statistically blocked by age and sex and assigned to receive either saline or tulathromycin at 1X, 3X, or 5X the recommended label dose for three times the normal duration. Goats were injected once per week for three weeks and necropsied one week following the third injection. No significant differences in physical parameters, feed intake, growth, clinical pathology measurements, or tissue (gross or histopathologic) assessments between treatment and control animals were identified that were associated with

tulathromycin administration. Results of this study indicate that tulathromycin would be a safe antimicrobial when used at the recommended label dose in caprines.

Introduction

Bacterial pneumonia is a common and often life-threatening respiratory problem in both meat and dairy goats (Washburn *et al*., 2007; Yener *et al*., 2009). *Pasteurella multocida* and *Mannheimia haemolytica*, often complicated by *Mycoplasma* species colonization, are the most common causes of respiratory infections in sheep and goats (Berge *et al*., 2006; Brogden *et al*., 1998; Washburn *et al*., 2007; Yener *et al*., 2009; Zamri-Saad & Mera, 2001). Options for approved antibiotic therapy to combat bacterial infections in caprines are severely limited and often must be administered in an off-label manner (Berge *et al*., 2006). The cephalosporin ceftiofur is the only antibiotic approved for use in goats (Fajt, 2001; Fajt, 2003; Washburn *et al*., 2007; Webb *et al*., 2004). Concern about antimicrobial resistance in human bacterial isolates and its association with cephalosporin administration to food animals has led to proposed limitations for extralabel cephalosporin use (Nolan, 2009). In addition, antimicrobials used successfully in other ruminant species available for extra-label administration under a valid veterinary-client-patient relationship may be unsuitable for goats. Tilmicosin, a macrolide antibiotic effective against respiratory pathogens when used in cattle and in sheep over 15kg, is cardiotoxic and potentially lethal in goats (Christodoulopoulos *et al*., 2002).

Tulathromycin is a novel triamilide antibiotic in the macrolide class shown to be safe and efficacious in treating respiratory disease in cattle and swine (Evans, 2005; Hart *et al*., 2006; Nowakowski *et al*., 2004). The objective of this study was to evaluate the safety of

tulathromycin in caprines using the youngest goats that would likely be treated for respiratory disease. The hypothesis was that there are no toxic effects in goats associated with tulathromycin treatment.

Materials and methods

Twelve male and 12 female mixed-breed (dairy and meat) goats, one to seven days of age weighing 3.6-7.3 kg, were enrolled in this study. Goats were identified by ear tag, housed individually in a climate-controlled facility, and fed a commercial, non-medicated milk replacer free choice. Animals were acclimated for nine days prior to initial injections and received no pre-treatment medications or vaccinations. All phases of the study were approved by the Institutional Animal Care and Use Committee of Iowa State University.

Daily physical exams and twice-daily clinical evaluations were performed on all animals from day -9 to day 21. Attitude, feed consumption, body temperature, heart rate, respiratory rate and fecal consistency were recorded. Pre-treatment blood, urine and fecal specimens were collected on day -2 to assess general health and obtain baseline clinical pathology data. Clinical pathology assessments were repeated on days 11 and 21.

Goats were statistically blocked by weight and sex and randomly assigned to treatment groups. Group 1 goats were control animals injected subcutaneously with 0.9% sodium chloride (Hospira, Inc., Lake Forest, Il). Groups 2, 3, and 4 goats were injected subcutaneously with 2.5-,7.5- or 12.5 mg/kg tulathromycin (Pfizer Animal Health, NY, NY), respectively, based upon body weights obtained the morning before dosing. Doses were equivalent to 1X, 3X, or 5X, of the label dose of tulathromycin approved for cattle.

Animals received one injection per week for three treatments (three times the recommended duration) at different predetermined locations on the neck.

On day 21 animals were weighed, euthanized by administration of IV xylazine to achieve general anesthesia and IV potassium chloride to induce cardiac arrest, and necropsied. Tissues were evaluated for gross and histopathologic changes that could be related to tulathromycin administration. Examined tissues included abomasum, adrenal glands, aorta, brain, cerebrum, thalamus, cerebellum, medulla oblongata, cecum, colon, duodenum, epididymis, esophagus, eyes (with optic nerve), gallbladder, heart (right atria, right ventricle, left atria, left ventricle), ileum, injection sites, jejunum, kidneys, liver (medial and left lobes), lung (peripheral tissue and large bronchus), mandibular, mediastinal, mesenteric lymph nodes, mammary gland, semimembranosus muscle, omasum, pancreas, sciatic nerve, pituitary, prostate, rectum, reticulum, rumen, salivary gland, spinal cord (cervical, thoracic, and lumbar sections), spleen, testes, ovaries, thymus, thyroid gland/parathyroid, urethra, urinary bladder, and uterus. Adrenal glands, brain, liver, kidneys, spleen, thyroid, thymus, and heart were weighed to ascertain organ to body weight ratios.

Blinding was maintained such that no person having knowledge of the dosing was involved in the clinical evaluations, necropsies or histopathologic exams. Quality assurance was provided in accordance with Good Laboratory Practices (GLP) (21 CFR Part 58) and was monitored by a Food and Drug Administration (FDA) representative to ensure adherence to GLP standards and the approved protocol.

Statistical evaluations of two- and three-way interactions (treatment-by-time, treatment-by-sex, and treatment-by-sex-by-time) at the 0.05 level of significance as well as main (treatment)

effects at the 0.10 level of significance (to meet FDA protocol specifications evaluating effects of tulathromycin administration) were performed using SAS statistical software (version 9.1, SAS Institute, Inc., Cari, NC). Baseline parameter information from the acclimation period was used as the covariate where applicable, and autoregressive covariate structure was used. Daily measurements of food consumption, body temperature, respiratory rate, and pulse rate were used to determined weekly means for statistical assessment. These weekly means, weekly body weights, and clinical pathology results were used in repeated measure analysis. Descriptive statistics were used to assess changes in gross and histopathologic tissue evaluations. Organ weights to body weight ratio comparisons were made using continuous variable analysis.

Results

Results of statistical comparison methods and results for physical and clinical pathology parameters are listed in Table 2.1; results of tissue assessments are listed in Table 2.2. All goats remained alert and responsive throughout the study. None of the signs of discomfort associated with tulathromycin injection described in cattle (head shaking, pawing) were identified in the goats following any of the injections, possibly due to lower injection volumes in these smaller juvenile study subjects. Two of the 5X goats vocalized briefly following injection and four goats (representing all treatment groups) had mild soft-tissue swelling at the injection site that resolved within 24-48 hours.

All groups had a slight decrease in mean feed intake during the week following the initial injection but mean consumption rebounded over the following weeks for all groups. Mean weekly feed consumption was lower for control goats (32.6 oz.) than for any of the treated

animals (34.5-36.3 oz.). All groups of goats gained weight over the course of the study. Heart rate and respiratory rates increased in all groups during the study.

PT and APTT were significantly different between treatment groups. Values were highest for control goats (mean PT 14.1 seconds, mean APTT 40.6 seconds; treatment goat mean PT 12.9 seconds, mean APTT 20.8 seconds) and differences were not attributed to tulathromycin treatment. Clotting profile normal ranges are not readily available for juvenile goats. All groups had PT levels higher than those considered normal for adult caprines but APTT levels were within normal limits for goats.

Treatment groups showed a significant difference in mean calcium level (10.1-10.9 mg/dl) from control animals (9.7-10.2 mg/dl), although all calcium values were within normal limits. Mean alkaline phosphatase levels were significantly higher (at $P < 0.05$) in the 1X (658.8 IU/L) and 3X (642.2 IU/L) groups than the control (419.4 IU/L) and 5X (456.7 IU/L) goats and not attributed to tulathromycin administration. Mean CK values were significantly different in the 5X (334.2 IU/L) group at the last measurement (after the third injection) from the other groups (187.0-246.0 IU/L); this variation may be associated with tissue irritation from larger drug volumes administered to this treatment group. Urine pH ranges were similar for all groups. Results of clinical pathology testing by treatment group are listed in Table 2.3.

No differences were observed in gross or microscopic exams of any tissues between the control and treated animals. Significant differences in thymus:body weight ratios were found between control and 3X goats but not with 1X or 5X goats. The 3X treatment group had a

higher mean thymic weight (94.4g) than all other groups (67.9-82.8g) which was not attributable to tulathromycin treatment.

Discussion

Washburn *et al.* (2007) demonstrated the safety of tulathromycin in adult goats at a single dose of 25 mg/kg (10 times the recommended dose for cattle and swine). Moderate, self-limiting post-injection pain and mild swelling at the injection sites were the only adverse effects identified in treated animals. The present study expanded the safety evaluation of this drug by 1) treating juvenile goats that would be expected to demonstrate detrimental effects related to tulathromycin if any were induced; 2) evaluating additional clinical pathology parameters and tissues; 3) using proposed label, 3X, and 5X doses to determine if any dose-related toxicity could be appreciated; and 4) repeating each treatment weekly for three weeks to assess any harmful outcomes from extended drug administration. No physical, clinical pathologic, or tissue differences associated with tulathromycin were detected between the control and treated animals.

The National Agricultural Statistics Service reported the U.S. meat goat inventory as of July 1, 2009 at 3.16 million head on 123,000 operations.[1] The lack of approved antimicrobials presents a risk to this species and may contribute to unsafe off-label drug use. Although minimum inhibitory concentration (MIC) breakpoints have not been defined for bacterial species from caprines, MIC studies on cattle respiratory pathogens indicate that tulathromycin could be a valuable antimicrobial against goat pathogens (Evans, 2005; Nowakowski *et al.,* 2004). The current study demonstrates the safety of tulathromycin

administration in this species, and further investigations into pharmacokinetics and efficacy are warranted.

Acknowledgements

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[1] USDA Economics, Statistics, and Marketing Information System, National Agricultural Statistics Service Report, July 24, 2009.

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Table 2.1. Parameters evaluated for differences and results between control goats and goats given 1X, 3X, and 5X of the label dose of tulathromycin weekly for three weeks.

Physical Parameter	Method of Evaluation	Significance
Body Temperature	Repeated Measures Analysis	NSD[a]
Body Weight	Qualitative Analysis	NSD
Respiratory Rate	Qualitative Analysis	NSD
Pulse Rate	Qualitative Analysis	NSD
Daily Milk Consumption	Qualitative Analysis	NSD
Hematology Parameters		
Red Blood Cells	Repeated Measures Analysis	NSD
Hemoglobin	Repeated Measures Analysis	NSD
Hematocrit	Repeated Measures Analysis	NSD
Mean Corpuscular Volume	Repeated Measures Analysis	NSD
Mean Corpuscular Hemoglobin	Qualitative Analysis	NSD
MCHC	Repeated Measures Analysis	NSD
Platelets	Repeated Measures Analysis	NSD
White Blood Cells	Repeated Measures Analysis	NSD
Total Neutrophils	Repeated Measures Analysis	NSD
Total Lymphocytes	Repeated Measures Analysis	NSD
Coagulation Parameters		
Prothrombin Time (PT)	Pair-wise comparisons	NSD
Activated Partial Thromboplastin Time (APTT)	Pair-wise comparisons	NSD
Chemistry Parameters		
Alanine Transaminase (ALT)	Qualitative Analysis	NSD
Sodium	Repeated Measures Analysis	NSD
Potassium	Repeated Measures Analysis	NSD
Chloride	Repeated Measures Analysis	NSD
Calcium	Qualitative Analysis	NSD
Phosphorus	Qualitative Analysis	NSD
Blood Urea Nitrogen (BUN)	Repeated Measures Analysis	NSD
Creatinine	Repeated Measures Analysis	NSD
Glucose	Repeated Measures Analysis	NSD
Total Protein	Repeated Measures Analysis	NSD
Albumin	Repeated Measures Analysis	NSD

[a]NSD= No significant difference between control and any treatment groups
[b]Significant difference between control and 1X, none between control and 3X or control and 5X
[c]Significant difference between control and 3X, none between control and 1X or control and 5X

Table 2.1 (continued)

Chemistry parameters	Method of Evaluation	Significance
Globulin	Repeated Measures Analysis	NSD
Albumin/Globulin ratio	Repeated Measures Analysis	NSD
Aspartate Transaminase (AST)	Repeated Measures Analysis	NSD
Creatine Kinase	Pair-wise comparisons	NSD
Alkaline Phosphatase	Pair-wise comparisons	Significant difference[b]
Gamma-glutamyl transferase (GGT)	Repeated Measures Analysis	NSD
Total Bilirubin	Repeated Measures Analysis	NSD
Cholesterol	Repeated Measures Analysis	NSD
Triglycerides	Repeated Measures Analysis	NSD
Sorbitol Dehydrogenase (SDH)	Repeated Measures Analysis	NSD
Urinalysis		
Urine SG	Repeated Measures Analysis	NSD
Urine Ph	Repeated Measures Analysis	NSD
Fecal Parasite Burden		
Fecal exam	Qualitative Analysis	NSD
Organ Weight Parameter		
Adrenal	Continuous variable analysis	NSD
Brain	Continuous variable analysis	NSD
Heart	Continuous variable analysis	NSD
Kidney	Continuous variable analysis	NSD
Liver	Continuous variable analysis	NSD
Spleen	Continuous variable analysis	NSD
Thymus	Qualitative Analysis	NSD
Thyroid	Continuous variable analysis	NSD
Organ:Body Weight Parameter		
Adrenal Gland:BW	Continuous variable analysis	NSD
Brain:BW	Continuous variable analysis	NSD
Heart:BW	Continuous variable analysis	NSD
Kidney:BW	Continuous variable analysis	NSD
Liver:BW	Continuous variable analysis	NSD
Spleen:BW	Continuous variable analysis	NSD
Thymus:BW	Pair-wise comparisons	Significant difference[c]
Thyroid:BW	Continuous variable analysis	NSD

Table 2.2 Gross and histopathologic tissues analyzed and results for analysis for differences between control and tulathromycin treatment groups.

Tissue Parameters	Gross exam	Histopathological exam
Abomasum	ND[a]	ND
Adrenals	ND	ND
Aorta (abdominal)	ND	ND
Brain		
Cerebrum	ND	ND
Thalamus	ND	ND
Cerebellum	ND	ND
Medulla oblongata	ND	ND
Cecum	ND	ND
Colon	ND	ND
Duodenum	ND	ND
Epididymis (if present)	ND	ND
Esophagus	ND	ND
Eyes (with optic nerve)	ND	ND
Gallbladder	ND	ND
Heart		
right atria	ND	ND
right ventricle	ND	ND
left atria	ND	ND
left ventricle	ND	ND
Ileum	ND	ND
Injection site		
first site	NSD[b]	NSD
other sites	ND	ND
Jejunum	ND	ND
Kidneys	ND	ND
Liver		
medial lobe	ND	ND
left lobe	ND	ND
Lung		
peripheral tissue	NSD	NSD
large bronchus	NSD	NSD
Lymph nodes		
mandibular	ND	ND
mediastinal	ND	ND
mesenteric	ND	ND

[a]ND=No abnormalities seen in control nor any treatment groups; no differences between groups
[b]NSD= No significant difference between control and any treatment groups using Fisher's Exact Test

Table 2.2 (continued)

Tissue Parameters	Gross Exam	Histopathologic Exam
Mammary gland (if present)	ND	ND
Muscle (semimembranosus)	ND	ND
Omasum	ND	ND
Pancreas	ND	ND
Peripheral nerves (sciatic)	ND	ND
Pituitary	ND	ND
Prostate (if present)	ND	ND
Rectum	ND	ND
Reticulum	ND	ND
Rumen	ND	ND
Salivary gland (mandibular)	ND	ND
Spinal cord		
cervical	ND	ND
thoracic	ND	ND
lumbar	ND	ND
Spleen	ND	ND
Testes (if present)	ND	ND
Ovaries (if present)	ND	ND
Thymus	ND	ND
Thyroid gland/parathyroid	ND	ND
Urethra	NSD	NSD
Urinary bladder	ND	ND
Uterus (if present)	ND	ND

Table 2.3. Results of clinical pathology examinations by treatment group in juvenile goats treated with saline or the label dose, three times the label dose, or five times the label dose of tulathromycin once weekly for three weeks.

	Control Group		1X Group		3X Group		5X Group	
Parameter	Mean	Range	Mean	Range	Mean	Range	Mean	Range
RBC ($x10^6$/ml)	14.8	9.2-19.4	13.4	9.1-18.8	13.0	9.3-18.4	15.9	9.7-20.2
HGB (gm/dl)	8.9	7.7-10.1	8.8	7.4-10.5	9.2	7.7-11.3	9.1	8.0-12.2
Hct (%)	29.1	24.2-34.3	29.9	25.5-33.5	31.8	25.5-38.3	29.8	26.4-42.7
MCV (fl)	20.5	14.3-36.3	23.5	15.1-35.6	25.6	18.2-39.1	19.2	14.2-38.4
MCH (pg)	5.8	4.1-10.5	6.8	4.8-10.1	7.3	5.6-10.6	5.8	4.6-10.6
MCHC (gm/dl)	30.5	27.5-32.5	29.5	26.7-31.7	29.1	27.4-32.0	30.5	27.5-32.8
Platelets ($x10^6$/ml)	1.2	6.3-1.2	1.1	6.9-20.0	1.1	4.8-18.2	1.1	7.6-17.7
WBC ($x10^3$/ml)	13.2	8.2-20.3	10.8	7.8-18.0	12.6	7.8-16.7	11.6	8.2-16.5
-Neutros ($x10^3$/ml)	7.2	2.5-13.6	5.4	2.3-10.8	5.6	2.8-7.6	4.3	3.0-11.2
-% Neutros (%)	53.1	26.2-68.0	43.8	25.6-71.2	45.1	17.8-58.5	47.6	23.8-66.1
-Lymphos ($x10^3$/ml)	5.4	1.8-11.0	6.1	2.2-7.1	6.4	2.4-11.9	7.0	3.8-9.2
-% Lymphos (%)	42.8	17.5-63.4	46.3	25.0-71.1	50.1	30.8-58.4	50.9	32.1-73.0
PT (sec)	14.1	11.9-18.8	13.6	11.5-19.5*	12.2	11.1-13.9*	12.8	11.1-16.8*
APTT (sec)	40.6	26.1-52.8	32.8	29.0-39.2*	30.6	22.7-36.5*	31.8	24.2-48.8*
ALT (IU/L)	8.7	4.0-13.0	8.9	5.0-12.0	8.3	4.0-12.0	9.6	5.0-14.0
Sodium (mEq/L)	145.6	143-147	146.4	144-148	146.3	144-149	145.8	144-148
Potassium (mEq/L)	5.5	4.8-6.2	5.4	4.3-6.4	5.5	4.8-6.1	5.4	5.0-6.1
Chloride (mEq/L)	109.0	106-112	108.5	106-114	109.7	105-117	108.1	105-111
Calcium (mg/dl)	10.0	8.8-10.9	10.4	10.0-11.0*	10.7	9.9-11.4*	10.3	9.8-11.0*
Phosphorus (mg/dl)	10.2	8.8-12.2	10.3	7.3-11.8	10.3	8.2-`12.4	10.5	8.7-12.4
BUN (mg/dl)	10.3	7.2-12.8	10.1	6.8-19.4	9.3	7.8-12.0	9.3	5.8-18.8
Creatinine (mg/dl)	0.4	0.3-0.7	0.4	0.3-0.5	0.5	0.3-0.6	0.4	0.3-0.5
Glucose (mg/dl)	77.8	13-104	97.4	63-131	87.9	42-111	89.5	62-109
Total Protein (gm/dl)	5.4	4.7-6.9	5.1	4.4-5.8	5.2	4.7-6.5	5.1	4.8-6.4
Alb (gm/dl)	2.7	2.2-3.2	2.7	2.2-2.9	2.8	2.2-3.1	2.7	2.4-3.2
Glob (gm/dl)	2.7	2.0-4.4	2.5	2.1-2.9	2.4	1.9-4.3	2.4	1.8-3.8

RBC (Red Blood Cell count), HGB (Hemoglobin), Hct (Hematocrit), MCV (Mean Corpuscular Volume),MCH (Mean Corpuscular Hemoglobin),MCHC (Mean Corpuscular Hemoglobin Concentration), Platelet number, WBC (White Blood Cell count), Neutros (total number and percent neutrophils), Lymphos (total number and percent lymphocytes, PT (Prothrombin Time), APTT (Activated Partial Thromboplastin Time), ALT (Alanine Transaminase), AST (Aspartate Transaminase), Sodium, Potassium, Chloride, Calcium, Phosphorus, BUN (Blood Urea Nitrogen), Creatinine, Glucose, Total Protein, Alb (Albumin), Glob (Globulin), Alb/Glob (Albumin/Globulin ratio), CK (Creatine Kinase), Alk Phos (Alkaline Phosphatase), GGT (Gamma-glutamyl transferase), Total Bili (Total Bilirubin), Cholesterol, Triglycerides, SDH (Sorbitol Dehydrogenase), urine SG (specific gravity), urine pH, and fecal floatation for parasites. * indicates significant difference at $P < 0.10$ between saline control and tulathromycin treated animals.

Table 2.3 (continued)

Alb/Glob ratio	1.1	0.6-1.5	1.1	0.8-1.4	1.2	0.5-1.6	1.2	0.7-1.8
AST (IU/L)	65.3	42-82	63.6	48-79	62.1	(41-78)	67.3	(48-84)
CK (IU/L)	229.5	157-391	213.6	103-481	180.9	93-277	266.8	106-543*
Alk Phos (IU/L)	419.4	231-655	658.8	296-1140*	642.2	299-1060	456.7	234-690
GGT (IU/L)	61.1	39-127	48.9	36-67	53.1	31-76	51.4	35-71)
Total Bili (mg/dl)	0.20	0.03-0.31	0.20	0.05-0.39	0.2	0.11-0.24	0.2	0.08-0.88
Cholesterol (mg/dl)	124.4	59-197	116.4	57-193	101.2	44-150	106.6	46-158
Triglycerides (mg/dl)	32.4	15-55	34.2	11-79	33.4	(9-72)	36.1	(12-112)
SDH (IU/L)	48.2	18.5-98.8	50.9	23.7-111	46.7	16.7-89.5	37.9	12.1-64.3
Urine SG	1.018	1.080-1.022	1.018	1.009-1.037	1.025	1.009-1.034	1.013	1.006-1.032
Urine pH	6.8	5.0-8.5	7.0	5.5-8.5*	5.8	5.5-7.5*	6.2	5.0-8.5*

CHAPTER 3. TULATHROMYCIN ASSAY VALIDATION AND TISSUE RESIDUES AFTER SINGLE AND MULTPLE SUBCUTANEOUS INJECTIONS IN DOMESTIC GOATS (*Capra aegagrus hircus)*

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Abstract

Tulathromycin is a macrolide antimicrobial labelled for the treatment of bacterial pneumonia in cattle and swine. A tulathromycin tissue analysis method utilizing tandem mass spectrometry that detects the common fragment of tulathromycin in these species was validated using goat tissues and evaluated by specificity, linearity, recovery, accuracy, precision, Limit of Detection (LOD), Limit of Quantification (LOQ), and stability over time. The assay was then used to study tissue depletion of tulathromycin in goats. In two different studies, six juvenile and ten market-age goats received a single injection of 2.5 mg/kg of tulathromycin subcutaneously; in a third study, 18 juvenile goats were treated with 2.5 mg/kg, 7.5 mg/kg, or 12.5 mg/kg tulathromycin weekly with 3 subcutaneous injections. Mean tulathromycin tissue concentrations were highest at injection site samples in all studies

and all doses evaluated. Lung tissue concentrations were greatest at day 5 (1.67 μg/g) and demonstrated dose-dependent increases over the doses studied. Concentrations were below the level of quantification (LOQ) in injection site and lung by day 18 and in liver, kidney, muscle, and fat at all time points. This study demonstrated that tissue levels in goats are very similar to those seen in swine and cattle.

Introduction

The Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994 (Food and Drug Administration, 1994) permits the use of certain approved animal and human drugs in an extralabel manner under a valid veterinary-client-patient relationship (Berge *et al*., 2006). Determination of appropriate withdrawal periods for this use is the responsibility of the prescribing veterinarian (Berge *et al*., 2006; Fajt, 2001; Fajt, 2003). Extrapolation of data from other species to estimate drug behavior may increase the likelihood of violative drug residues in products for human consumption.

Bacterial pneumonia is a frequent health problem in caprines, with *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida*, and *Mycoplasma* spp. implicated as the most common bacterial causes (Ackermann & Brogden, 2000; Berge *et al*., 2006; Brogden *et al*., 1998; Washburn *et al*., 2007; Yener *et al*., 2009; Zamri-Saad & Mera, 2001). Ceftiofur is the only antimicrobial presently labeled in this species (Fajt, 2001; Fajt, 2003; Washburn *et al*., 2007; Webb *et al*., 2004); however, cephalosporins have some limitations. Successful treatment requires daily administration, which may be difficult to accomplish under field conditions. Concerns over cephalosporin resistance in human bacterial strains may lead to potential restrictions on their use in food animals (Nolen, 2009). Additionally,

cephalosporins are not active against *Mycoplasma* species which are important pathogens of goats (Rosenbusch *et al*., 2005b).

Macrolide antibiotics rapidly dissemination from plasma to lung tissues making them useful therapeutics against bacterial pneumonia (Williams & Sefton, 1993). Newer macrolides display enhanced lung penetration and extended tissue half lives (Benchaoui *et al*., 2004). Tulathromycin, a macrolide labeled to treat bacterial respiratory disease in cattle and swine, provides a seven day treatment with a single injection, indicating that it could be a useful therapeutic agent in caprines (Benchaoui *et al*., 2004; Evans, 2005; Nowakowski *et al*., 2004).

Tulathromycin is manufactured as a single isomer or parent compound, designated CP-472,295(e). This molecule equilibrates in solution into in 9:1 mixture of two tulathromycin isoforms, designated CP-472,295 and 547,272, respectively (Gáler *et al*., 2004). The common fragment or marker residue for these two different isoforms is CP-60,300, and the FDA-approved regulatory methodology detects this common fragment. An alternative method of tulathromycin evaluation published by Gáler *et al* (2004) involves establishing the levels of the parent compound 472,295(e) and is primarily used to measure overall tulathromycin concentrations in plasma or lung tissue samples.

The present study was conducted to 1) validate these methods of tulathromycin detection in caprine tissues and 2) to determine tissue depletion of tulathromycin over time in goats to provide data useful in avoiding drug residues in the human food supply.

Materials and methods

Analytical method for quantifying levels of CP-60,300 in tissues

The method used was developed by Pfizer Animal Health Group and is described in the Pfizer Methods 1535N-60-99-294 and 1525N-60-99-175 (NADA, 141-244, 2005). Analysis for the common fragment CP-60,300 was conducted in liver, kidney, muscle, fat, and injection site samples using a method provided by Pfizer, Inc. Briefly, tulathromycin residues were converted to the marker residue by acid-catalyzed hydrolysis with 2 N hydrochloric acid at 60° C. The supernatant was passed through an OASIS® MCX (Waters, Milford, MA) cation exchange column and eluted with acetonitrile:ammonium hydroxide (95:5). The eluate was evaporated to dryness at 50° C under a gentle stream of nitrogen (N-Evap, Organomation Associates, Inc, Berlin, MA) and reconstituted with the aqueous mobile phase. Quantification was by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

Samples were injected on an Acquity® UPLC (Waters, Milford, MA); injection volume was 5 µL. The mobile phase was 0.02 M ammonium acetate, pH 4.0 with formic acid-acetonitrile (77:23) at a flow rate of 0.3 mL/min. An Ace C8, 2.1 X 50 mm, 3 µm (MacMod, Chadds Ford, PA) column was maintained at 35° C. The mass spectrometer was a Thermo TSQ Quantum Discovery Max (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization source operated in the positive ion mode. Ions were monitored in the selective reaction monitoring mode for the common fragment and an internal standard (structurally similar compound provided by Pfizer) with transitions from 577.2 to 420.2 and 591.3 to 434.2 respectively.

Validation of the analytical method for quantifying CP60,300 in caprine tissues

Method proficiency in bovine tissues, specificity, linearity, recovery, accuracy, precision, Limit of Detection (LOD), Limit of Quantification (LOQ), stability in the final extracts, and stability of tulathromycin after 3 freeze / thaw cycles. The complete analytical procedure was applied to blank sample specimens for each tissue (liver, muscle, kidney, fat, and injection site). Method proficiency was evaluated by assaying identical sets of spiked bovine and caprine tissues as well as incurred bovine tissue samples. Specificity of the assay was considered correct if no other peak was detected at the same retention time as the marker residue (CP-60,300) on blank sample chromatograms. The linearity of the method, the response ratio (CP-60,300 peak area / internal standard peak area) vs. concentration, was evaluated after analysis of the five calibration lines generated during the variation trial. The calibration ranges were 25 to 250 $ng.mL^{-1}$ and 75 to 750 $ng.mL^{-1}$. Linearity was calculated by the method of least squares linear regression of the response ratio vs. the theoretical concentrations, and had a tissue equivalent linearity of 0.6 to 36.0 µg/g. In goat tissue, the average extraction recovery was established for concentrations of 2.75, 5.5 and 11.0 $\mu g.g^{-1}$ of liver, 1.125, 2.5 and 4.5 $\mu g.g^{-1}$ of muscle and fat, and 7.5, 15 and 30.0 $\mu g.g^{-1}$ of kidney. Each spike level was analyzed in triplicate five times for an n=15. Intraday and interday precision and accuracy of the analytical procedure were evaluated after replicate analyses (n = 3) of control goat liver, kidney, muscle and fat spiked at three concentration levels (1/2-, 1- and 2-times the safe concentration for liver, kidney and muscle and low, medium and high concentrations for fat). Using the blank samples (n = 15) from each tissue, the LOD was defined at the mean of the noise + 3 times the standard deviation of the mean; the LOQ was defined as the mean of the noise + 10 times the standard deviation of the mean. The noise is

determined at the retention time of the CP-60,300 peak. Short-term stability was evaluated in the acid extract and final extract after analysis of control goat liver spiked with tulathromycin and stored at room temperature or refrigerated. All samples were analyzed at 0, 1, 2, 3, 4, 7, 10 and 14 days, and stability was assessed as the coefficient of variation between the standard deviation and the observed concentrations of CP-60,300. The stability of tulathromycin in goat liver was evaluated after three freeze / thaw cycles (frozen for at least 18 hours at -80˚C, thawed to room temperature).

Lung samples were assayed for the parent compound of tulathromycin designated CP-472,295(e) according to the protocol of Gáler *et al*. (2004) using ultra-high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Briefly, after aqueous dilution with internal standard, the samples were extracted by solid phase extraction (Bond-Elut 200 mg CBA, Varian, Lake Forest, CA). The analyte was eluted with 5:95 ammonium hydroxide-acetonitrile and evaporated to dryness (N-Evap, Organomation Associates, Inc, Berlin, MA). Samples were reconstituted with mobile phase (82:18 0.02 M ammonium acetate pH 4.0 with formic acid-acetonitrile) and injected (5 µL) on the UPLC (Waters, Milford, MA). The flow rate was 0.25 mL/min. The column was an Ace C8, 2.1 X 50 mm, 3 µm (MacMod, Chadds Ford, PA) maintained at 35° C. The mass spectrometer was a Thermo TSQ Quantum Discovery Max (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization source. Positive ions were monitored in the selected reaction monitoring mode with the doubly charged precursor → product ion pair of 404.1 → 578.0. The assay was linear from 2 to 500 ng/mL (r^2 = 0.9883) and had a limit of detection of 0.7

ng/mL and a limit of quantification of 2.0 ng/mL. Inter-assay and intra-assay variation as measured by %RSD were 11.1% and 11.3%, respectively. The recovery was 99.6% ± 6.7%.

Animal tulathromycin studies

Three tissue residue studies were conducted in different populations of goats. All animals were acclimated prior to the study; identified by ear tags; housed individually; and fed ad-lib hay with goat starter ration (single-dose study) or ad-lib milk-replacer (multi-dose study). Single-dose subjects were injected with tulathromycin (Draxxin®, Pfizer Animal Health, NY, NY) subcutaneously on the right side of the neck; goats receiving three doses were injected in three different subcutaneous locations. Doses were based on body weights collected the day before injection. An equal volume of sterile 0.9% sodium chloride (Hospira, Inc., Lake Forest, IL) was injected to control animals.

Pre-study physical examinations were within normal limits and daily observations on physical attitude, appetite, and fecal consistency were recorded. All phases of the studies were approved by the Institutional Animal Care and Use Committee of Iowa State University or the Institutional Animal Care and Use Committee of Veterinary Resources, Inc., Ames. IA.

Liver, kidney, muscle, fat, lung, and injection site samples were collected, immediately frozen, and stored at -80^{o}C until shipment for analysis. Homogenized tissue samples from untreated animals were spiked with tulathromycin, frozen, and analyzed to ensure freezer stability of the samples throughout the storage period.

In study 1, three male and three female, seven- to eight-week-old mixed-breed meat and dairy goat kids weighing 7.7-9.5 kg were randomly assigned to tissue collection time points

and injected with 2.5mg/kg tulathromycin subcutaneously. Goats were humanely euthanized with IV xylazine to achieve general anesthesia and IV potassium chloride to induce cardiac arrest, and tissues were collected: two goats at 14 days; two goats at 21 days; one goat at 28 days; and one goat at 35 days post-injection.

In study 2, eleven dairy-mixed breed and 19 Boer, five-to six-month-old, male (intact and castrated) goats weighing 13.8-27.4 kg were blocked by weight and randomly assigned to one of six treatment groups: five groups injected with 2.5 mg/kg tulathromycin with various tissue collection time points and one saline control group. Goats were humanely euthanized via a captive bolt and exsanguinated on day 5, day 12, day 18, day 27, and day 48 following tulathromycin injection. One randomly selected control goat was euthanized at each time point.

Study 3 was conducted to determine if multiple injections or different dosages affected the pharmacokinetics of tulathromycin. Twelve male and twelve female, two- to three-week-old, 3.4-8.3 kg, mixed-breed meat and dairy goat kids were blocked by sex and weight and randomly assigned to control or one of three treatment groups receiving 2.5mg/kg, 7.5mg/kg, or 12.5 mg/kg tulathromycin once weekly for three consecutive weeks. Seven days after the third injection, goats were humanely euthanized with IV xylazine to achieve general anesthesia and IV potassium chloride to induce cardiac arrest, and tissues were collected.

Mean, standard deviation, and coefficient of variation of tulathromycin concentrations were calculated for each tissue type. Differences in tissue tulathromycin between treatment and control market-age goats as well as overall and dose-normalized tulathromycin concentrations in the multi-dose treatment groups were evaluated for significance at $P < 0.05$

using a mixed model assessing least squares means differences with SAS statistical software (version 9.1, SAS Institute, Inc., Cari, NC). A substitution method using ½ LOD for values <LOD was used for statistical comparisons. A further assessment of this substitution method using the non-parametric Kruskal-Wallis test for overall tissue differences was conducted to evaluate for bias due to the left-censored data associated with values <LOD. Although substitution methods for values <LOD are not ideal, non-parametric analysis attempts to minimize bias associated with unknown values below LOD.

Results

Method validation

No endogenous compounds co-eluting with CP-60,300 were observed in chromatograms of 15 blank control goat liver and muscle samples. The retention time of CP-60,300 was approximately 1.0 min. The limit of detection for liver, muscle, kidney, fat, and injection site were 0.75, 0.24, 0.29 0.14, and 0.24 µg/g respectively, while the limit of quantifications were 1.91, 0.69, 1.66, 0.61, and 0.69µg/g. Inter- and intra- assay variation as measured by percent relative standard deviation (%RSD) were 4.8 and 12.7 for liver, 7.6 and 20.9 for kidney, 11.3 and 28.6 for muscle and 4.8 and 10.7 for fat. Recoveries were 113.6% for liver, 112.6% for kidney, 100.1% for muscle and 98.5% for fat. Short-term stability percent CV ranged from 2.5 to 11.3 and percentage of difference between the samples after 3 freeze / thaw cycles and prior to freezing ranged from 7.0 to 22.5in liver tissue. The response ratio did not show a degradation trend over the time frames analyzed.

Tissue sample analysis

Results of tissue concentrations of tulathromycin for juvenile goats given a single injection are listed in Table 3.1. Tulathromycin CP-30,600 was detected only in injection site samples and concentrations demonstrated variability, with a 20-fold difference between the two goats sampled on day 14 and a 7-fold difference at day 21. Injection site concentrations were below the LOD by day 35 post-injection and no CP-30,600 was detected in any other tissues.

Mean tissue concentrations from the market-age goats are shown in Table 3.2. At five days post-injection, only lung tissue (1.67 µg/g of CP-472,295[e]) and injection sites (7.62 µg/g of CP-30,600) had mean tissue levels over the limit of quantification (LOQ) and had detectable residues in all animals tested. Lung tissue tulathromycin concentrations also had the least variability in all tissues examined at all time points. Injection site samples had the greatest mean values of all tissues at day 5 and day 12; values had decreased to 1.27 µg/g by day 18. Mean CP-30,600 levels were below the LOQ in liver, kidney, muscle, and fat at each time point and in all tissues by day 18. Control goats had no detectable tulathromycin in any tissues. Tissue tulathromycin concentrations were not significantly different ($P < 0.05$) between control and treated animals in any of the examined tissues by the end of the study but did differ in lung tissue (CP-472,295[e]), liver (CP-30,600), fat (CP-30,600), and injection site (CP-30,600) over time. No significant differences ($P < 0.05$) were detected between control and treated goats by day 18 in any of the examined tissues.

Table 3.3 lists the tulathromycin tissue concentrations in juvenile goats given weekly doses for three treatments and the *P* values for the differences among doses. Increasing dose resulted in the most dramatic tissue level increase of CP-30,600 in liver tissue; values

increased 385% from 2.5 mg/kg (0.7 µg/g) to 7.5 mg/kg (3.40 µg/g) and another 43% from 7.5 mg/kg to 12.5 mg/kg (4.87 µg/g). Tulathromycin concentration in other tissues demonstrated smaller increases with increasing dose, ranging from 36% of CP-30,600 in injection site samples in the two highest doses (7.5 mg/kg, 17.9 µg/g; 12.5 mg/kg, 24.4 µg/g) to 163%of CP-472,295(e) in lung samples (2.5 mg/kg, 0.72 µg/g; 7.5 mg/kg, 1.9 µg/g). Since liver, kidney, muscle, and fat had undetected CP-30,600 concentrations in the 2.5 mg/kg treated animals, percent increases are difficult to determine in these tissues.

Mean tissue concentrations normalized by dose are shown in Figure 3.1. Dose-normalized tissue levels of CP-30,600 were significantly different ($P < 0.05$) for liver between the 2.5 mg/kg (0.28 mg/g), and 7.5 mg/kg groups (0.45 mg/g) but not between 2.5 mg/kg and 12.5 mg/kg (0.39 mg/g) groups. Differences ($P < 0.05$) in the concentrations of the common fragment also existed in kidney, muscle, and fat tissue by dose between the 2.5 mg/kg animals (<LOD) and the two higher dose groups. No significant ($P < 0.05$) differences were found in these tissues between the two higher dose group animals, nor between any of the treatment groups in lung tissue or injection sites. No tulathromycin was detected in any of the tissues from the saline-treated controls.

Discussion

The different methods for tulathromycin detection have been described for various matrices with different goals. Since tissue residues detected in food animal species that have been treated with a specific drug are used by the FDA to establish adequate withdrawal periods after drug administration, detection protocols that have received regulatory approval must be used to evaluate these samples. The method used to identify levels of the common

fragment CP- 60,300 was designed to detect concentrations of any of the tulathromycin isoforms; consequently, it gives the most stringent assessment of drug residues in tissues from treated animals. Sample matricies for this method include those involved in drug absorption or elimination and/or those that may end up in the human food supply, which in this study include liver, kidney, fat, muscle, and injection site samples.

Target tissue concentrations can be used in evaluating certain drugs for efficacy (Benchaoui *et al*., 2004; Gáler *et al*., 2004; Nowakowski *et al*., 2004). This is particularly true for macrolide antimicrobials due to their rapid distribution out of plasma and into tissues. An accurate, precise, and robust detection method is needed to characterize a drug's distribution in the animal and its relationship to efficacy (Gáler *et al*., 2004). Consequently, a detection method that focuses on the most active structure of the drug being evaluated provides more information on the potential therapeutic potential of the specific medication than overall tissue concentrations. The method described by Gáler *et al* (2004) focuses on the evaluation of tulathromycin in lung tissues and plasma since these matricies are critical to determining potential drug efficacy in the tissue(s) being targeted for therapy. This method identifies the parent compound of tulathromycin, CP-472,295(e), and attempts to take advantage of the sensitivity of tandem mass spectrometry while minimizing pre-analysis sample extraction and processing (Gáler *et al*., 2004).

Tissue tulathromycin levels (CP-60,300 in liver, kidney, muscle, fat, and injection site; CP472,295[e] in lung in goats are similar to those seen in cattle and swine; results from all three species are shown in Figure 3.2. Mean lung concentration in cattle at 6 days post SC injection was 3.01 mg/g and in swine at 7 days post intra-muscular (IM) injection was 1.44

mg/g (Benchaoui *et al*., 2004; Nowakowski *et al*., 2004). Similar persistence in the lung was identified in goats (1.7 mg/g at 5 days) and lung was the only sample matrix that had significantly greater concentrations between control and treated market-age goats on day 5 and 12, suggesting that tulathromycin is potentially a useful drug for the treatment of bacterial respiratory infections in this species. Values of the common fragment at the injection site at days 5 and 12 were consistently higher in goats compared with cattle and swine, but were consistently lower by day 18 and throughout the remainder of the study. Values of CP-60,300 in liver, kidney, and muscle tissue at all time points were consistently lower in goats than in cattle and swine, and were below the LOQ at each collection date. Since the majority of subjects had tulathromycin concentrations below the LOQ, no true depletion patterns could be ascertained; however, results of this study do provide information on tissue disposition and removal of tulathromycin over time in goats.

Tulathromycin has been shown to be very effective against major respiratory pathogens of both cattle and swine (Hart *et al*., 2006; Kilgore *et al*., 2005; Rooney *et al*., 2005; Sweeney *et al*., 2008). The major benefit of macrolide antimicrobials is the persistence in tissues, providing sustained antibiotic levels for effective treatment. *M. Haemolytica. B. trehalosi,* and *P. multocida* are frequent pathogens associated with pneumonia in goats. While no minimum inhibitory concentrations have been established for these bacteria in goats, using guidelines on breakpoints in cattle can give information on tulathromycin susceptibility in these agents. A review of cases submitted to the Iowa State University Veterinary Diagnostic Laboratory demonstrated 95.5% (21/22) of *M. haemolytica* isolates, 100% of *B. trehalosi* (3/3), and 100% (5/5) of *P. multocida* isolates were susceptible to

tulathromycin with mode MIC values of 8μg/ml, 4 μg/ml, and 2μg/ml, respectively. Using lung tulathromycin (CP-472,295[e]) concentrations for the first 18 days, the calculated AUC for this period (AUC_{last}) was 523.2 μg*hr/ml. For modern macrolide antimicrobials, determination of total pathogen exposure to a given antimicrobial frequently is used to estimate potential efficacy of a specific drug against a specific bacterial pathogen (Evans, 2005; McKellar *et al*., 2004). AUC/MIC ratios have been used as potential indicators of efficacy since they incorporate both time and concentration effects to estimate this exposure (Evans, 2005). In goats, the AUC/MIC ratio for the first 18 days after injection was *M. haemolytica* is 65.4, for *B trehalosi* is 130.8, and for *P. multocida* was 261.6; ratios of 25 or greater are indications of efficacy in this pharmacokinetic/pharmacodynamic relationship.

Results of the present study indicate that tissue distribution of tulathromycin is similar in goats to that seen in cattle and swine. Interestingly, tissue concentrations in the juvenile goats were below the LOD for all tissues except injection site, while detectable levels were found in the older animals. This may indicate that there is a drug tissue transport process or protein binding present in the market age goats that has not yet developed in very young. Since the majority of subjects had tulathromycin concentrations below the LOQ, no true depletion patterns could be ascertained; however, results of this study do provide information on tissue disposition and removal of tulathromycin over time in goats. Further investigations into this drug in the caprine species are warranted to evaluate its usefulness in the treatment of bacterial respiratory disease in goats.

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Table 3.1. Tissue tulathromycin concentrations (µg/g) from juvenile goats receiving a single subcutaneous tulathromycin injection of 2.5 mg/kg by day post-injection.

	Day 14		Day 21		Day 28	Day 35		
Tissue	# 457	# 460	# 448	# 458	# 452	# 438	LOQ	LOD
Liver	<LOD	<LOD	ND	<LOD	<LOD	ND	1.91	0.75
Kidney	ND	ND	ND	ND	ND	ND	1.66	0.29
Muscle	ND	ND	ND	ND	ND	ND	0.69	0.24
Fat ND	ND	ND	ND	ND	ND	ND	0.61	0.14
Injection site	16.8	0.81	14.3	2.11	7.7	0.25*	NE	NE

Lung	ND	ND	ND	ND	ND	ND	NE	NE

LOD = limit of detection; ND = none detected; * = below limit of quantification (LOQ); NE = not established

Table 3.2. Mean tissue tulathromycin levels (µg/g) from market-age goats receiving a single subcutaneous tulathromycin injection of 2.5 mg/kg by day post-injection.

	Day 5 (n=5)			Day 12 (n=5)			Day 18 (n=5)			Day 27 (n=5)			Day 48 (n=5)		
Tissue	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
Liver*	1.28[e]	0.32	24.8[a]	1.18[e]	0.42	36.0[c]	<LOD			<LOD			<LOD		
Kidney	<LOD			<LOD			0.51[e]	0	0[d]	0.43[e]	0.04	8.4[b]	0.31[e]	0.01	3.2[b]
Muscle	0.24[e]	0	0[d]	<LOD			<LOD			<LOD			<LOD		
Fat*	0.21[e]	0.08	35.2[b]	0.15[e]	0	0[d]	<LOD			<LOD			<LOD		
Inj. site*	7.62	5.65	74.2	16.5	12.2	73.9[c]	1.27	0	0[d]	NS			NS		
Lung*	1.67	0.24	14.4	0.64	0.13	20.2	0.23[e]	0.10	45.2	0.15[e]	0.04	27.9[a]	<LOD		

Inj. Site = injection site; LOD = limit of detection; NS = no sample; [a] one animal <LOD; [b] two animals <LOD; [c] three animals <LOD; [d] four animals <LOD; [e] = below limit of quantification (LOQ); * = significantly different concentrations over time from control animals at $P < 0.05$; all control goats had tulathromycin levels <LOD in all tissues

Table 3.3. Mean tulathromycin tissue levels (µg/g), SD, CV%, and *P*-values for differences between dose groups from juvenile goats receiving one injection per week of the associated dose for three weeks. Tissues were collected seven days after third injection.

	Dose									
	2.5 mg/kg (n=6)			7.5 mg/kg (n=6)			12.5 mg/kg (n=6)			
Tissue	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	*P*-value
Liver	0.70[e]	0.41	57.6[a]	3.40	0.80	23.7	4.87	0.88	18.1	< 0.0001[xyz]
Kidney	<LOD			1.65	0.64	39.2	3.28	0.40	12.1	< 0.0001[xyz]
Muscle	0			0.65[e]	0.34	52.4[b]	1.33	0.68	51.3	0.0025[yz]
Fat	0			0.36[e]	0.23	64.5	0.65[e]	0.33	50.9	< 0.0001[xyz]
Injection site	8.76	7.44	85.0	17.9	8.66	48.4	24.4	8.98	37.8	0.0008[xy]
Lung	0.72	0.14	19.5	1.80	0.69	38.3	3.39	1.13	33.2	0.0001[xyz]

LOD = limit of detection; [a] one animal <LOD; [b] two animals <LOD; [c] three animals <LOD; [d] four animals <LOD; [e] = below limit of quantification (LOQ); [x] significantly different between 2.5 mg/kg and 7.5 mg/kg dose groups; [y] significantly different between 2.5 mg/kg and 12.5 mg/kg dose groups; [z] significantly different between 7.5 mg/kg and 12.5 mg/kg dose groups

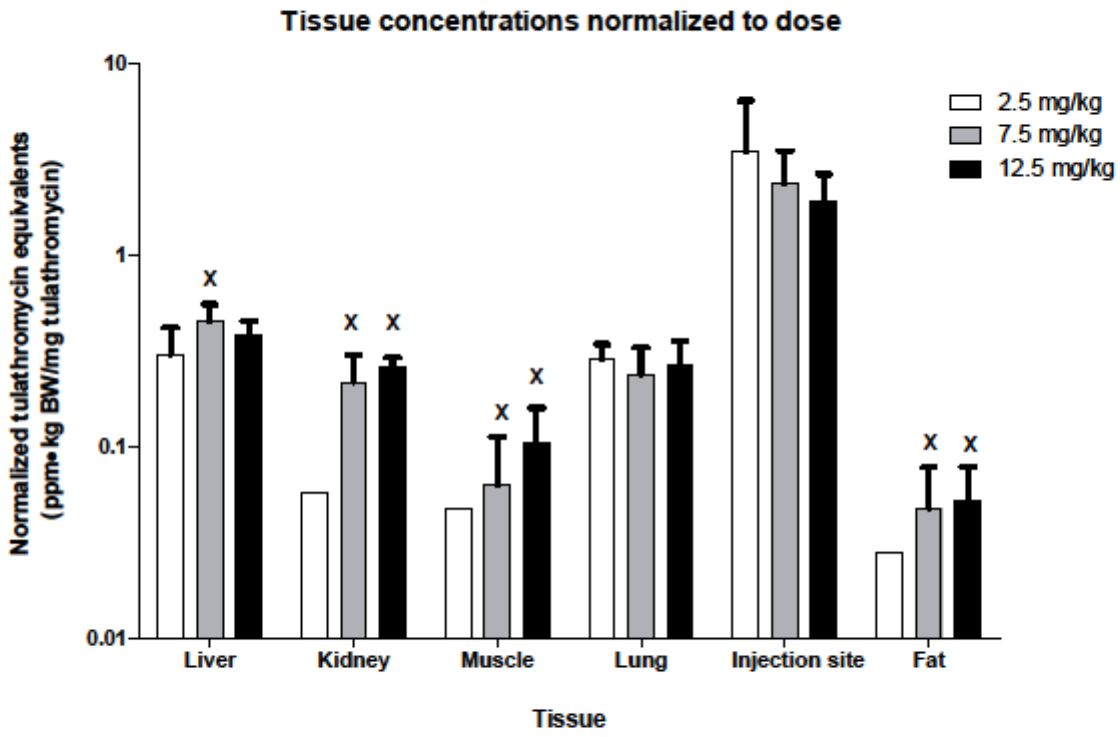

Figure 3.1. Dose-normalized tissue tulathromycin concentrations following administration of multiple subcutaneous doses of tulathromycin in goats (*Capra aegagrus hircus*, n=6 per group) at doses of 2.5, 7.5 or 12.5 mg/kg. "X" indicates statistically significant difference from the 2.5 mg/kg treatment group ($P < 0.05$)

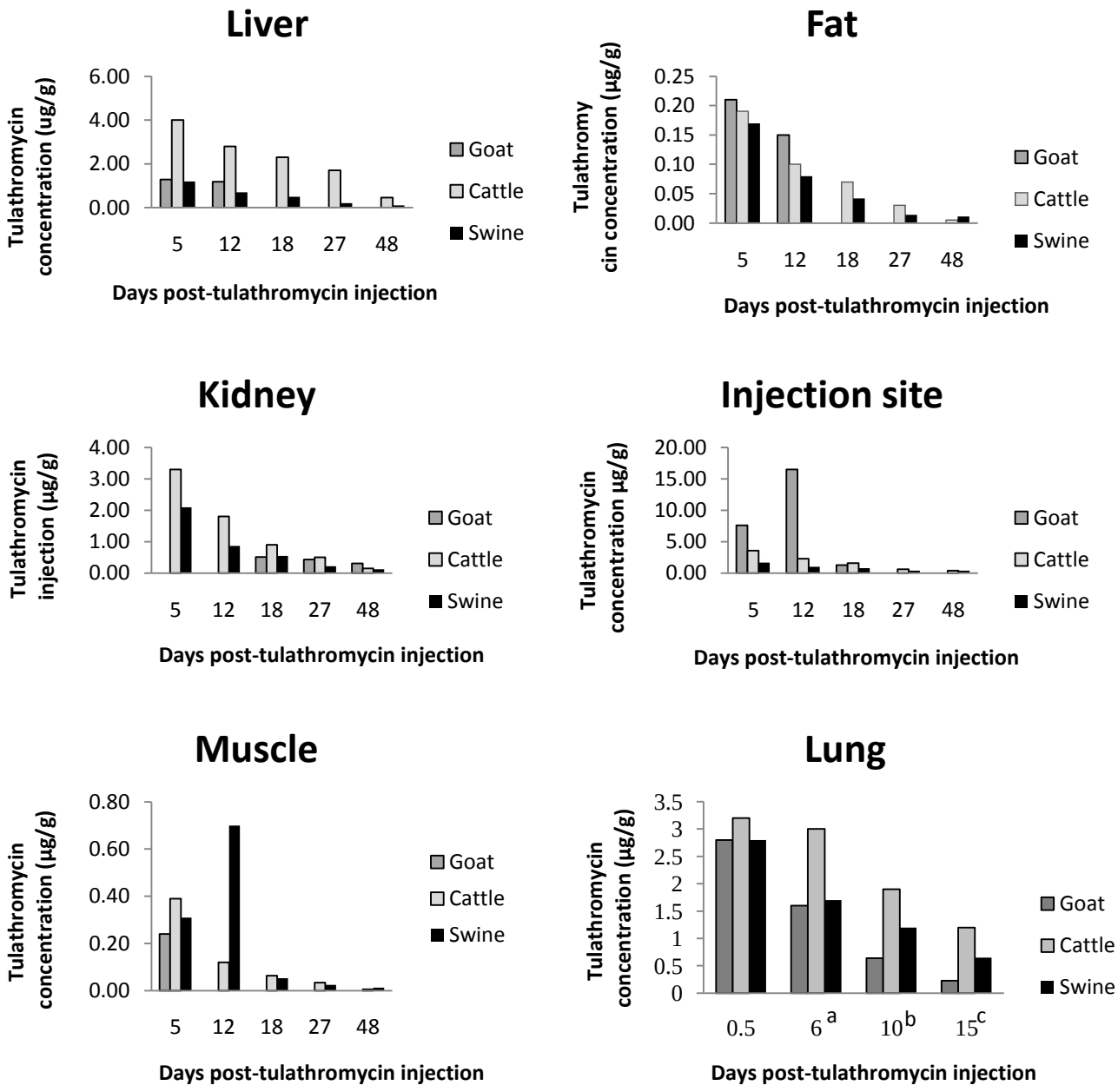

Figure 3.2. Mean tissue tulathromycin levels (µg/g) from tissues of goats, cattle, and swine receiving a single subcutaneous tulathromycin injection of 2.5 mg/kg by day post-injection. Liver, kidney, muscle, fat, and injection site samples were assayed for the common fragment of tulathromycin, CP-60,300. Lung samples were assayed for the parent compound of tulathromycin, CP-472,295 (e). [a] goat lung tissues collected on day 5; [b] goat lung tissues collected on day 12; [c] goat lung tissues collected on day 18

CHAPTER 4. PHARMACOKINETICS OF TULATHROMYCIN AFTER SINGLE AND MULTIPLE SUBCUTANEOUS INJECTIONS IN DOMESTIC GOATS (*Capra aegagrus hircus)*

Modified from a paper submitted to the in *The Journal of Veterinary Pharmacology and Therapeutics*

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Abstract

Tulathromycin, a novel triamilide in the macrolide class, is labeled for treatment of bacterial pneumonia in cattle and swine. The present manuscript evaluates pharmacokinetics of tulathromycin in goats. In two different studies, six juvenile and ten market-age goats received a single injection of 2.5 mg/kg of tulathromycin subcutaneously; in a third study, 18 juvenile goats were treated with 2.5 mg/kg, 7.5 mg/kg, or 12.5 mg/kg tulathromycin weekly with 3 subcutaneous injections. Pharmacokinetic parameters estimated from the plasma concentrations from single injections were similar between the two groups of goats and to previously reported parameters in cattle and swine. Mean terminal half lives were 59.1±7.6 and 61.2±8.7 h and apparent volumes of distribution were 21.6±7.1 and 29.0±7.1 L/kg for juvenile and market age goats, respectively. In the multi-dose study, dose-corrected AUC_{∞}, apparent clearance, terminal half life, and apparent volume of distribution demonstrated

significantly differences at $P < 0.05$ among repeated injections but not among doses. Overall, pharmacokinetic parameters in goats are similar to those reported in cattle and swine, and tulathromycin may prove a useful drug for treating respiratory disease in goats.

Introduction

Bacterial pneumonia is a frequent cause of morbidity and mortality in the caprine species (Washburn *et al*., 2007; Yener *et al*., 2009). The most common bacterial species associated with caprine pneumonia include *Mannheimia haemolytica*, *Bibersteinia trehalosi, Pasteurella multocida,* and *Mycoplasma* spp.(Ackermann & Brogden, 2000; Berge *et al*., 2006; Brogden *et al*., 1998; Washburn *et al*., 2007; Yener *et al*., 2009; Zamri-Saad & Mera, 2001).

Drugs that are FDA-approved for use in goats are severely limited, and often must be administered in an extra-label manner (Berge *et al*., 2006). Currently ceftiofur is the only labeled antimicrobial available (Fajt, 2001; Fajt, 2003; Washburn *et al*., 2007; Webb *et al*., 2004). Due to the concern over increased cephalosporin resistance in human bacterial strains and its potential association with use of this antibiotic in food animals, restrictions may be imposed on use of cephalosporins in food animals (Nolen, 2009). Additionally, *Mycoplasma* species are important pathogens of goats and cephalosporins are not expected to be active against these bacteria (Rosenbusch *et al*., 2005). The choice of a suitable antimicrobial to be used off label in goats must often be made based on clinical experience or on data collected in other species.

Tulathromycin is a novel triamilide antimicrobial in the macrolide class shown to be safe and effective in cattle and swine to treat bacterial respiratory disease (Benchaoui *et al*., 2004;

Evans, 2005; Nowakowski *et al*., 2004). Macrolide structure facilitates rapid distribution of these drugs from the blood stream into tissues (Williams & Sefton, 1993). Newer macrolides, such as azithromycin and tulathromycin, have increased lung tissue uptake and longer half lives than older macrolides such as erythromycin (Benchaoui *et al*., 2004). In addition, the triple-ionized form of tulathromycin produces displacement of the Mg^{2+} ions present in the outer cell wall of gram negative bacterial pathogens, facilitating drug entry into these agents (Evans, 2005). Tulathromycin demonstrated a 90% reduction in bacterial number (MIC_{90}) against the following agents collected from diseased tissues in cattle: *M. haemolytica* at 2 µg/ml; *P. multocida* at 1 µg/ml; and *M. bovis* at 1µg/ml (Evans, 2005). Since goats are susceptible to many of the same bacterial agents as cattle, tulathromycin could be a useful therapeutic agent in the caprine species. The present study was conducted to establish pharmacokinetic behavior of tulathromycin in goats.

Materials and methods

Study Design

Three studies were conducted in different populations of goats. All animals were acclimated prior to the initiation of the study; were identified by individual ear tags; were housed individually; and were fed ad-lib hay with goat starter ration (single-dose study) or milk-replacer (multi-dose study). Animals receiving a single dose of tulathromycin (Draxxin®, Pfizer Animal Health, NY, NY) were injected on the right side of the neck; goats receiving three doses were injected in a different location for each injection. Doses were based on body weights collected the day before injection. Single-dose studies were not randomized since all animals received the same dose. Results of pre-study physical

examinations were within normal limits and daily observations on physical attitude, appetite, and fecal consistency were recorded. All phases of the studies were approved by the Institutional Animal Care and Use Committee of Iowa State University and Veterinary Resources, Inc. Blood samples were collected into sterile glass tubes containing lithium heparin as an anticoagulant; centrifuged within one hour of collection; transferred to a sterile cryovial; and stored at -80^{o}C until shipment for analysis. Plasma samples obtained prior to treatment were spiked with tulathromycin to establish freezer stability standards and analyzed to ensure stability of the samples throughout the storage period.

Single-Dose study—juvenile goats

Three male and three female, six week old mixed-breed meat and dairy goat kids with a mean body weight of 8.4 kg (range =7.7-9.5 kg) were injected with 2.5mg/kg tulathromycin subcutaneously. Plasma samples were collected from the jugular vein prior to injection, and at 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, and 360 hr following injection.

Single-Dose study—market age goats

Five dairy-mixed breed and five Boer, six month old, male (intact and castrated) goats with a mean body weight of 19.9 kg (range = 17.2-27.4 kg) received one subcutaneous injection of 2.5 mg/kg tulathromycin. Blood samples were collected from the jugular vein prior to injection, and at 1, 2, 4, 6, 8, 10 min; and 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, and 360 hr following injection

Multi-dose study—juvenile goats

The multi-dose study was conducted to evaluate effects of multiple injections using three different dosages of tulathromycin. Nine male and nine female, one to two week old mixed-breed meat and dairy goat kids with a mean body weight of 5.15 kg (range = 3.4-8.3 kg) were blocked by sex and weight and randomly assigned to one of three treatment groups receiving 2.5mg/kg, 7.5mg/kg, or 12.5 mg/kg tulathromycin once a week for three weeks. Blood samples were collected from each animal prior to the first injection, at 4, 52, 100, and 148 hr after each of the first two injections and 4, 52, 100, and 164 hours after the third injection.

Tulathromycin Analysis

Persons performing tulathromycin analysis were blinded to information on treatment group in the multi-dose study. Samples were assayed for tulathromycin according to the protocol of Gáler *et al*. (2004) using ultra-high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Briefly, after aqueous dilution with internal standard, the samples were extracted by solid phase extraction (Bond-Elut 200 mg CBA, Varian, Lake Forest, CA). The analyte was eluted with 5:95 ammonium hydroxide-acetonitrile and evaporated to dryness (N-Evap, Organomation Associates, Inc, Berlin, MA). Samples were reconstituted with mobile phase (82:18 0.02 M ammonium acetate pH 4.0 with formic acid-acetonitrile) and injected (5 µL) on the UPLC (Waters, Milford, MA). The flow rate was 0.25 mL/min. The column was an Ace C8, 2.1 X 50 mm, 3 µm (MacMod, Chadds Ford, PA) maintained at 35° C. The mass spectrometer was a Thermo TSQ Quantum Discovery Max (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization source. Positive ions were monitored in the selected reaction monitoring mode with the doubly

charged precursor → product ion pair of 404.1 → 578.0. The assay was linear from 2 to 500 ng/mL (r^2 = 0.9883) and had a limit of detection of 0.7 ng/mL and a limit of quantification of 2.0 ng/mL. Inter-assay and intra-assay variation as measured by %RSD were 11.1% and 11.3%, respectively. The recovery was 99.6% ± 6.7%.

Pharmacokinetic analysis

Plasma tulathromycin concentrations in the juvenile and market-age goats following the single dose of tulathromycin were analyzed for each individual animal by compartmental and non-compartmental approaches (NCA) with the software WinNONLIN® version 5.2.1 (Pharsight Corporation, Mountain View, CA). Biexponential equations for a two-compartment open model $Cp = Ae^{-\alpha t} + Be^{-\beta t}$ (where A and B are y-intercept constants, α is the rate constant of the distribution phase, and β is the rate constant of the elimination phase) were used to fit the observed data. Weighting of the data using the inverse square of the concentration was used to improve the line fits and residual plots. The goodness of fit of the data with the model was determined by visual examination of the line fits, residual plots, and Akaike's information criteria (Yamakoa *et al*., 1978). Because the first sample obtained in the juvenile goats was the observed C_{max}, only data points after the C_{max} in the market age goats were used in the comparison.

In addition, non-compartmental analyses (NCA) were performed on the individual goat plasma concentrations for both groups of goats from the single dose studies and the juvenile goats from the multi-dose study. Calculated non-compartmental parameters include the area under the time-concentration curve (AUC) from time 0 to infinity (AUC_∞); the area under the curve divided by the dose (AUC_∞/D); the mean transit time (MTT); apparent whole body

clearance (CL/F); the terminal elimination phase rate constant and its half life (λ_z and $t_{½\,\lambda z}$); and the apparent volume of distribution (Vz/F). The maximum plasma concentration (C_{max}) and the time to maximum concentration (T_{max}) were collected directly from the data.

For the multi-dose study, WinNonLin would normally use the trough concentration, which would be taken immediately prior to the next consecutive dose for use in estimating NCA parameters. Due to technical constraints a trough concentration was not obtained prior to the 2nd and 3rd doses in this study. To determine if the lack of a trough concentration significantly affected the pharmacokinetic results, a comparison was made among the WinNonLin-estimated AUC_∞ for each dosing interval (1st, 2nd and 3rd dose) with the initial plasma concentration for the 2nd and 3rd dose set to an upper limit of the last measured concentration at 24 hr prior to next dose (AUC_{upper}) versus when the initial dose was set to zero (AUC_{lower}) by default in WinNonLin. The percentage difference in the AUC was calculated as

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The actual AUC_∞ will be somewhere between the two estimates; therefore if the percentage differences between AUC_{upper} and lower AUC_{lower} was small, then the lack of an actual trough sample was assumed to not affect the pharmacokinetic analysis significantly.

Statistical analysis

Mean, standard deviation (SD) and coefficient of variation were calculated for each parameter in each treatment group. Results of pharmacokinetic parameters were assessed for differences between study groups using the Students t-test with SAS statistical software

(version 9.2, SAS Institute, Inc., Cary, NC). Parameters for each goat from the multi-dose groups were evaluated for significant differences ($P < 0.05$) from either dose level or injection repetition by a Two-Way ANOVA (Prism for Windows v 5, GraphPad Software, Inc., La Jolla, CA). A Bonferroni post hoc test was used to determine significant differences among specific doses and repetitions, if the ANOVA showed statistically significant effects.

Results

Single dose

Individual animal results as well as mean, standard deviation (SD), and coefficient of variation (C V) from compartmental and non-compartmental analysis for juvenile and market age goats that received a single subcutaneous tulathromycin injection are listed in Tables 4.1 and 4.2, respectively. The comparison of the compartmental fits of the elimination phase with the observed plasma concentrations for the juvenile and market age goats are shown in Figures 4.1A and 4.1 B, respectively. For the majority of the juvenile goats and the market age goats, the WinNonLin-defined terminal phase was used for calculation of the NCA pharmacokinetic parameters. However for goats 452 and 458, WinNonLin included only 3 or 4 time points to estimate the terminal phase, and the estimated half lives were greater than the time period during which they were calculated. Therefore for those 2 goats the terminal phase was selected for a period that maximized R^2 and also included at least 5 data points and was at least 3 times greater than the estimated half life. For the market age goats, the WinNonLin-selected terminal period included only 3 points for goats 28, 35, and 77. For that reason the NCA pharmacokinetic parameters were estimated by a user-defined terminal period according to the criteria described for the juvenile goats.

Mean values, SD, and *P* values derived from assessing the groups for differences using the Student t-test are listed in Table 4.3. Significant differences ($P < 0.05$) were found between the groups for the NCA parameters mean AUC_{∞} (juvenile-10580 ng-h/ml; market-age 7918 ng-h/ml) and mean CL/F (juvenile-250.3 nl-kg/hr; market-age 327.7 nl-kg/hr); and the compartmental parameters mean α (juvenile-0.62 hr^{-1}; market-age 0.41 hr^{-1}) and mean $t_{½\alpha}$ (juvenile-1.20 hr; market-age 2.01 hr). No other parameters were significantly different between the groups. AUC_{∞} values from the compartmental and non-compartmental models were within 10% of each other for all of the juvenile goats and eight of the ten market age goats. Mean β phase values from the compartmental model for the juvenile and market age animals (0.012 and 0.011 hr^{-1}, respectively) were comparable to the λ_z values calculated from the non-compartmental model (0.012 and 0.011 hr^{-1}, respectively).

Multi-dose

The estimated terminal phase for each injection at all three dose levels from the multi-injection study are shown in Figure 4.2., and non-compartmental pharmacokinetic parameters are shown in Figure 4.3. The differences in the AUC_{∞} calculated with an initial plasma concentration of zero versus the upper limit of the concentration were all less than 1%; with the highest at 0.65% (data not shown). Therefore the pharmacokinetic parameters were compared for estimates from the WinNonLin default concentration of zero for the initial plasma concentration at the time of injection for the multiple doses.

Assessment of pharmacokinetic parameters in the multi-dose animals detected several unique findings. While mean AUC_{∞} increased slightly greater than the increase in dose among the dose levels, the dose-normalized AUC_{∞} were not significantly different among

doses at $P < 0.05$ (Figure 4.3A). At the highest dose of 12.5 mg/kg, the dose-normalized AUC_{∞} was significantly lower ($P < 0.05$) for the second and third injections compared with the first injection. Terminal half life was significantly higher ($P < 0.05$) for all dose groups between the second and third injections and between the first and third injections in the highest dose group (Figure 4.3 B). For all dose levels apparent clearance, CL/F, was significantly higher ($P <0.05$) from injection 3 compared with injection 1, and from injection 2 compared to injection 1 for the two higher dose (7.5 mg/kg and 12.5 mg/kg) groups (Figure 4.3 C). There was a statistically significant difference at $P < 0.05$ in apparent volume of distribution, Vz/F, for injection 3 compared with injection 1 for all doses. In addition, Vz/F was significantly greater between injections 2 and 3 for the lowest dose (2.5 mg/kg) group and between injections 1 and 2 for the highest dose (12.5 mg/kg) group (Figure 4.3 D). The concentration measured at 4 hr post administration was the highest measured concentration following each injection for all 3 dose levels. The dose-normalized C_{max} decreased with repeated injections for all 3 dose levels, but the decrease was only statistically significant ($P < 0.05$) at the highest dose of 12.5 mg/kg (Figure 4.3 E). This decrease in C_{max} with repeated injection contributed to the injection-dependent decrease in AUC_{∞}. No differences in MTT were noted between doses or repeated injections (Figure 4.3 F).

Discussion

The pharmacokinetic parameters of tulathromycin following a single subcutaneous injection in goats in this study demonstrated similar ranges and variability to those reported in cattle (Gáler *et al*., 2004; Nowakowski *et al*., 2004) and swine (Benchaoui *et al*., 2004). Mean C_{max} reported for animals administered 2.5 mg/kg ranged from 300 to 1300 ng/ml in

cattle and from 585 to 1100 ng/ml for swine; the mean C_{max} in this study was 988 ng/ml for the juvenile goats and 1185 ng/ml for the market age goats. One limitation to the previous data in cattle and swine and to the juvenile goats in this study was the absence of an absorption phase during the time points collected for determination of the plasma concentrations of tulathromycin. This study adjusted the sampling times to collect more frequent samples at early time points in the market age goats, and therefore, the observed T_{max} (mean of 13.3 min) and C_{max} (1185 ng/ml) are more accurate estimates of the peak time and concentration of tulathromycin. The reported plasma AUC_{∞} ranged from 11,200 to 18,700 ng/ml for cattle, and 9000 to 15,600 ng/ml for swine compared with a mean ± SD of 10,580 ± 2743 ng/ml for the single-dose juvenile goats and 7918 ± 1594 ng/ml for market age goats in this study. Terminal phase half life for a single administration of tulathromycin have been reported to be 87 to 110 hours for plasma samples in cattle and 39.8 to 91 hours in swine compared with the estimates of 59.1 hours in juvenile goats, and 61.2 hours in market age goats in this study. Variability such as that seen in these studies is not unexpected and had been reported in pharmacokinetic research, especially with subcutaneous routes of administration (Riviere, 1999).

The terminal phase half lives measured in the market age goats in this study are shorter than a recent study ($t_{½}$ 117.9 ±33.9 hr), which also analyzed the pharmacokinetics of tulathromycin following a single dose of 2.5 mg/kg administered subcutaneously in 5-7 month old goats (Young *et al*., 2010). In that study, however, sampling was conducted for a longer duration (456 hours post administration) and a different analytical technique was used. In the present work, a shorter sampling period was used with more frequent collections

during the absorption phase, which influenced the calculation of the half life. In both of these tulathromycin studies in goats, sampling periods contained at least 3 half lives but not a more ideal period of 5-7 half lives. In addition, the percentage of the AUC_{∞} that was extrapolated was small, with a mean 3.4% and 3.0% for the adult goats from the Young *et al*. (2004) study and market age goats from this study, respectively.

The half lives for tulathromycin from the juvenile goats in this study following a single dose were longer than those estimated for the multi-dose study. The data from the multi-dose study was sparse due to experimental limitations and samples were collected for only 7 days after each injection; therefore the estimated half lives most likely do not represent the actual terminal phase. The time period for the estimate was consistent among injections and dose levels so evaluation for effects of dose or repeat on the pharmacokinetics of tulathromycin in juvenile goats is valid. Of note from the multi-dose study was the significant increase in the apparent clearance and volume of distribution with repeated injections. This was a result of the decrease in dose-normalized AUC_{∞} due to lower C_{max} values. Since with extravascular administration, only the apparent clearance and volume of distribution can be calculated, the increase may reflect decreased bioavailability of the administered tulathromycin. An increase in elimination could result in a lower C_{max} and an increase in apparent clearance, however, the terminal half life was not significantly lower with each repeated injection. In addition, C_{max} may have been missed by four hours post-injection. The large value of Vz/F for all three dose levels indicated extensive peripheral distribution for tulathromycin in the goats. Experimental design did not allow a trough sample to be collected immediately prior to the next tulathromycin injection, which would be

ideal. To verify that the lack of the trough sample did not affect the statistical comparison of the AUC_{∞} and other parameters calculated with AUC_{∞}, an upper limit of the concentration measured at 24 hours prior to the subsequent injection was used. The increase in the AUC_{∞} would be less than 1 %, which is significantly less than the 33% decrease in the dose-normalized AUC_{∞} with repeated injection.

Apparent clearance was significantly lower in juvenile goats than in the market-age animals. Although no information is currently available on the metabolism of tulathromycin, clearance of other long-acting macrolides are primarily through bile elimination with a small percentage excreted unchanged in the urine (Farrington, 1998; Williams & Sefton, 1993). It had been shown that in both animals and humans production and activity of bile acids is limited early in life and improves around weaning (Kussaibati *et al*., 1982; Pettigrew and Moser, 1991; Watkins, 1985). The juvenile goats studied here were two weeks old at study initiation and five weeks old at study termination, and diminished bile acid metabolism may have influenced drug clearance in these animals, which would also account for the greater mean value for AUC_{∞} in juvenile goats over market age subjects.

There currently are limited antimicrobials available for treatment of bacterial infections in goats, which are increasingly being used in food-production. Pharmacokinetic data on potential drugs is critical to ensure effective treatment and safe food for human consumption. Tulathromycin has been shown to be very effective against major respiratory pathogens of both cattle and swine (Godinho *et al*., 2005; Hart *et al*., 2006; Kilgore *et al*., 2005; Schunicht *et al*., 2007). Pharmacokinetic data on potential drugs is critical to ensure effective treatment and safe food for human consumption. Results of this study indicate that

tulathromycin behaves similarly in goats to cattle and swine and may represent a useful therapeutic in the caprine species.

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Table 4.1. Compartmental and non-compartmental pharmacokinetic parameters for juvenile goats (*Capra aegagrus hircus* ; 8 weeks of age) given a single 2.5 mg/kg subcutaneous injection of tulathromycin. CL/F and V/F were compared as estimates of clearance and volume of distribution, respectively, since no IV administration was performed.

	Compartmental parameters						
Animal	A (ng/ml)	B (ng/ml)	α hr^{-1}	β hr^{-1}	AUC_{∞} (ng-hr/ml)	$t_{½\ \alpha}$ (hr)	$t_{½\ \beta}$ (hr)
438	671.9	162.1	0.63	0.012	14166	1.11	56.0
448	1254.1	117.1	0.90	0.012	11403	0.77	59.3
452	709.9	95.1	0.72	0.012	9199	0.97	59.8
457	905.9	87.1	0.55	0.011	9270	1.25	60.7
458	594.2	59.7	0.48	0.010	6986	1.45	66.7
460	464.4	120.8	0.42	0.013	10575	1.64	54.4
Mean	766.7	107.0	0.62	0.012	10266	1.20	59.5
SD	279.4	34.9	0.17	0.001	2428	0.32	4.3
CV%	36.4	32.7	28.4	7.0	23.7	26.6	7.2

	Non-compartmental parameters								
Animal	AUC_{∞} (ng·hr/ml)	AUC_{∞}/D (ng-h-kg/ml/mg)	MTT (hr)	CL/F (ml/hr/kg)	λ_z (hr^{-1})	$t_{½\ \lambda z}$ (hr)	Vz/F (L/kg)	C_{max} (ng/ml)	T_{max} (hr)
438	15004	6002	70.5	166.6	0.013	54.2	13.0	880.7	0.25
448	11601	4640	72.3	215.4	0.013	55.3	17.2	1322.0	0.25
452	9391	3756	73.5	266.2	0.009	73.8	28.4	1007.0	0.25
457	9597	3839	70.1	260.4	0.012	59.4	22.3	1179.0	0.25
458	6792	2717	71.9	368.1	0.012	58.9	31.3	793.1	0.25
460	11095	4438	65.0	225.3	0.013	52.7	17.1	744.3	0.25
Mean	10580	4232	70.6	250.3	0.012	59.1	21.6	987.7	0.25
SD	2743	1097	3.0	67.9	0.001	7.6	7.1	226.8	0.25
CV%	25.9	25.9	4.2	27.1	11.2	13.0	33.0	23.0	0.25

A, distribution intercept; B, elimination intercept; α, distribution constant; β, elimination constant; AUC_{∞}, area under the plasma concentration vs. time curve extrapolated to infinity; $t_{½\ \alpha}$, distribution half-life; $t_{½\ \beta}$, elimination half-life; AUC_{∞}/D, area under the plasma concentration vs. time curve extrapolated to infinity/Dose; MTT, mean transfer time; CL/F, apparent clearance; λ_z, elimination rate constant; $t_{½\ \lambda z}$, elimination half-life; Vz/F, apparent volume of distribution; C_{max}, observed maximum plasma concentration; T_{max}, time to observed maximum plasma concentration

Table 4.2. Compartmental and non-compartmental pharmacokinetic parameters in market aged goats (*Capra aegagrus hircus* ; 20 weeks of age) given a single 2.5 mg/kg subcutaneous injection of tulathromycin. CL/F and V/F were compared as estimates of clearance and volume of distribution, respectively, since no IV administration was performed.

	Compartmental parameters						
	A	B	α	β	AUC_∞	$t_{½\,\alpha}$	$t_{½\,\beta}$
Animal	(ng/ml)	(ng/ml)	hr^{-1}	hr^{-1}	(ng*hr/ml)	(hr)	(hr)
10	414.4	100	0.32	0.011	10024	2.16	60.3
13	906.1	59.3	0.27	0.012	8355	2.57	58.4
28	195.1	40.8	0.16	0.009	5646	4.38	75.0
30	766.9	43.1	0.43	0.009	6654	1.62	78.2
35	644.1	129	0.47	0.012	12168	1.47	58.1
36	867.3	96.1	0.53	0.012	9634	1.30	57.7
39	926.3	53.5	0.62	0.009	7413	1.12	76.6
67	686.0	82.5	0.50	0.014	7376	1.40	50.4
77	352.4	86.6	0.24	0.012	8510	2.90	56.3
78	1063.0	98.2	0.57	0.013	9507	1.22	53.9
Mean	682.2	78.9	0.41	0.011	8528	2.01	62.5
SD	282.0	28.8	0.15	0.002	1888	1.03	10.1
CV%	41.3	36.4	37.7	15.0	22.1	51.0	16.2

	Non-compartmental parameters								
	AUC_∞	AUC_∞/D	MTT	CL/F	λ_z	$t_{½\,\lambda z}$	Vz/F	C_{max}	T_{max}
Animal	(ng·hr/ml)	(ng*h*kg/mL/mg)	(hr)	(ml/hr/kg)	hr^{-1}	(hr)	(L/kg)	(ng/ml)	(hr)
10	10731	4292	74.8	233.0	0.012	56.5	19.0	924.5	0.25
13	7301	2921	55.6	342.4	0.010	72.0	35.5	1265.0	0.17
28	5342	2137	67.9	468.0	0.012	56.6	38.2	325.0	0.25
30	6769	2708	77.7	369.3	0.010	70.3	37.5	1304.0	0.17
35	8984	3594	74.4	278.3	0.009	76.2	30.6	1529.0	0.25
36	8901	3560	66.2	280.8	0.012	56.6	22.9	1123.0	0.17
39	7308	2923	75.7	342.1	0.011	61.3	30.3	1520.0	0.25
67	6750	2700	61.7	370.4	0.013	54.7	29.2	1296.0	0.25
77	7471	2988	65.7	334.6	0.012	56.0	27.0	576.3	0.17
78	9578	3831	62.0	261.0	0.014	48.7	18.3	1988.0	0.25
Mean	7914	3165	68.2	328.0	0.011	60.9	28.8	1185.0	0.22
SD	1600	640	7.3	68.1	0.001	8.9	7.1	481.8	0.04
CV%	20.2	20.2	10.7	20.8	13.8	14.6	24.7	40.7	19.8

A, distribution intercept; B, elimination intercept; α, distribution constant; β, elimination constant; AUC_∞, area under the plasma concentration vs. time curve extrapolated to infinity; $t_{½\,\alpha}$, distribution half-life; $t_{½\,\beta}$, elimination half-life; AUC_∞/D, area under the plasma concentration vs. time curve extrapolated to infinity/Dose; MTT, mean transfer time; CL/F, apparent clearance; λ_z, elimination rate constant; $t_{½\,\lambda z}$, elimination half-life; Vz/F, apparent volume of distribution; C_{max}, observed maximum plasma concentration; T_{max}, time to observed maximum plasma concentration

Table 3. Mean, SD and T-test *P*-values for non-compartmental and compartmental pharmacokinetic parameters calculated from plasma tulathromycin concentrations following administration of a single 2.5 mg/kg subcutaneous injection of tulathromycin in juvenile (n=6) and market age (n=10) goats (*Capra aegagrus hircus)*. CL/F and V/F were compared as estimates of clearance and volume of distribution, respectively, since no IV administration was performed.

Pharmacokinetic	Juvenile goats		Market Age goats		t-test
Parameter	Mean	SD	Mean	SD	*P*- value
AUC_{∞} (ng*h/mL)	10580	2743	7918	1594	0.030
AUC_{∞}/D (ng*h*kg/mL/mg)	4232	1097	3167	638	0.030
MTT (hr)	70.6	3.0	68.3	7.1	0.985
CL/F (nl/hr/kg)	250.3	67.9	327.7	67.9	0.053
λ_z (hr^{-1})	0.012	0.002	0.011	0.001	0.995
$t_{½\ \lambda z}$ (hr)	59.1	7.6	61.8	8.7	0.529
V/F (L/kg)	26.4	16.4	31.4	14.5	0.146
C_{max} (ng/ml)	987.8	226.9	1185	481.8	0.367
A (ng/ml)	766.7	279.4	682.2	282.0	0.570
B (ng/ml)	107.0	34.9	78.9	28.8	0.102
α (hr^{-1})	0.62	0.17	0.41	0.15	0.027
β (hr^{-1})	0.012	0.001	0.011	0.002	0.633
AUC_{∞} (ng*h/mL)	10266	2428	8529	1888	0.131
$t_{½\ \alpha}$ (hr)	1.20	0.32	2.01	1.03	0.082
$t_{½\ \beta}$ (hr)	59.5	4.27	62.5	10.1	0.504

$AUC_{\infty,}$ area under the plasma concentration vs. time curve extrapolated to infinity; AUC_{∞}/D, area under the plasma concentration vs. time curve extrapolated to infinity/Dose; MTT, mean transfer time; CL/F, apparent clearance; $\lambda_{z,}$ elimination rate constant; $t_{½\ \lambda z}$, elimination half-life; Vz/F, apparent volume of distribution; C_{max}, observed maximum plasma concentration; T_{max}, time to observed maximum plasma concentration

A, distribution intercept; B, elimination intercept; α, distribution constant; β, elimination constant; $AUC_{\infty,}$ area under the plasma concentration vs. time curve extrapolated to infinity; $t_{½\ \alpha}$, distribution half-life; $t_{½\ \beta}$, elimination half-life

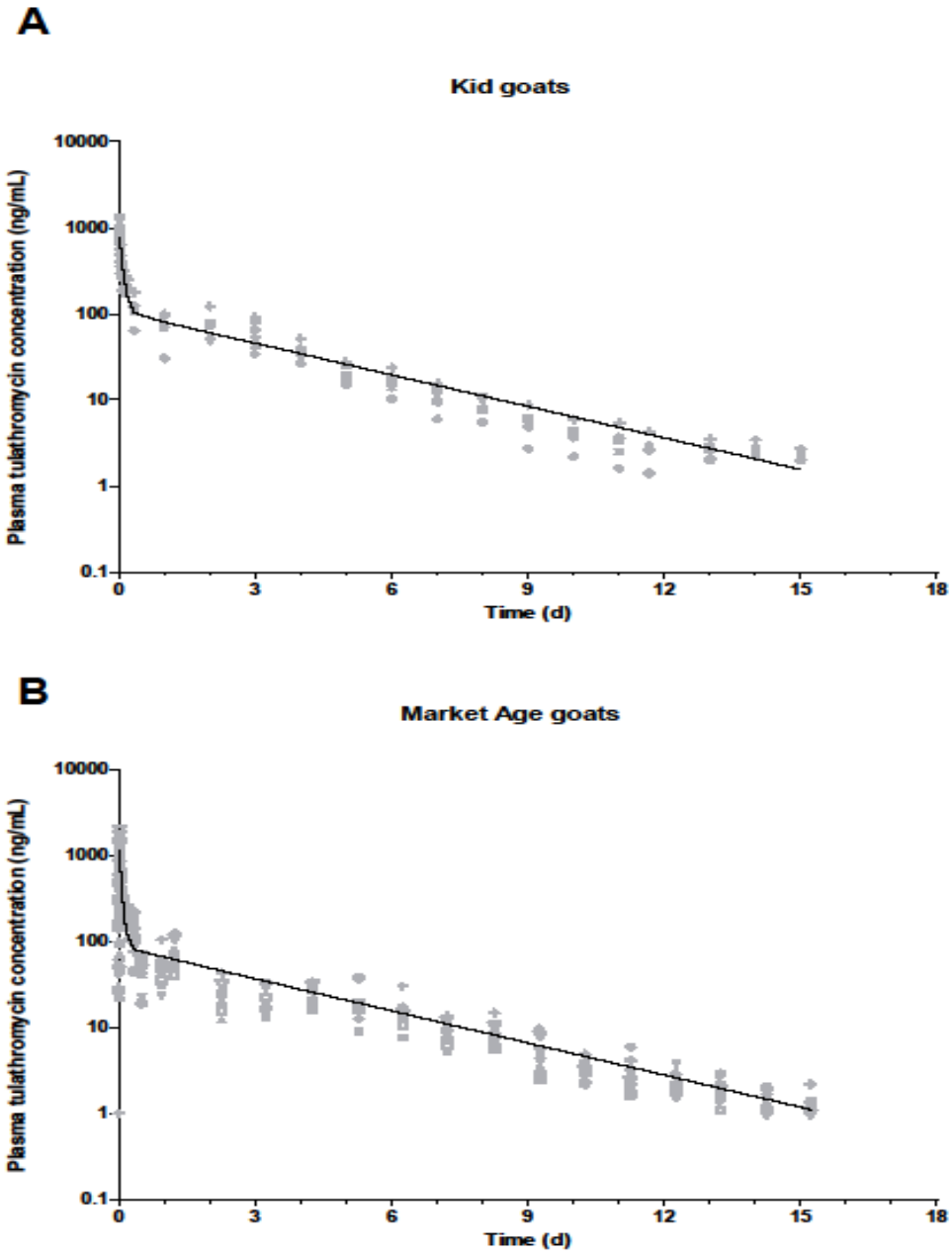

Figure 4.1. Plasma concentrations (symbols) of tulathromycin for A) individual juvenile goats (*Capra aegagrus hircus* ; 8 weeks of age) and B) market aged goats (*Capra aegagrus hircus* ; 20 weeks of age) given a single 2.5 mg/kg subcutaneous injection of tulathromycin plot against the mean predicted tulathromycin (line) during the elimination phase. The compartmental parameters used to generate the predicted concentration are given in Tables 1 and 2. Each symbol represents values from one animal.

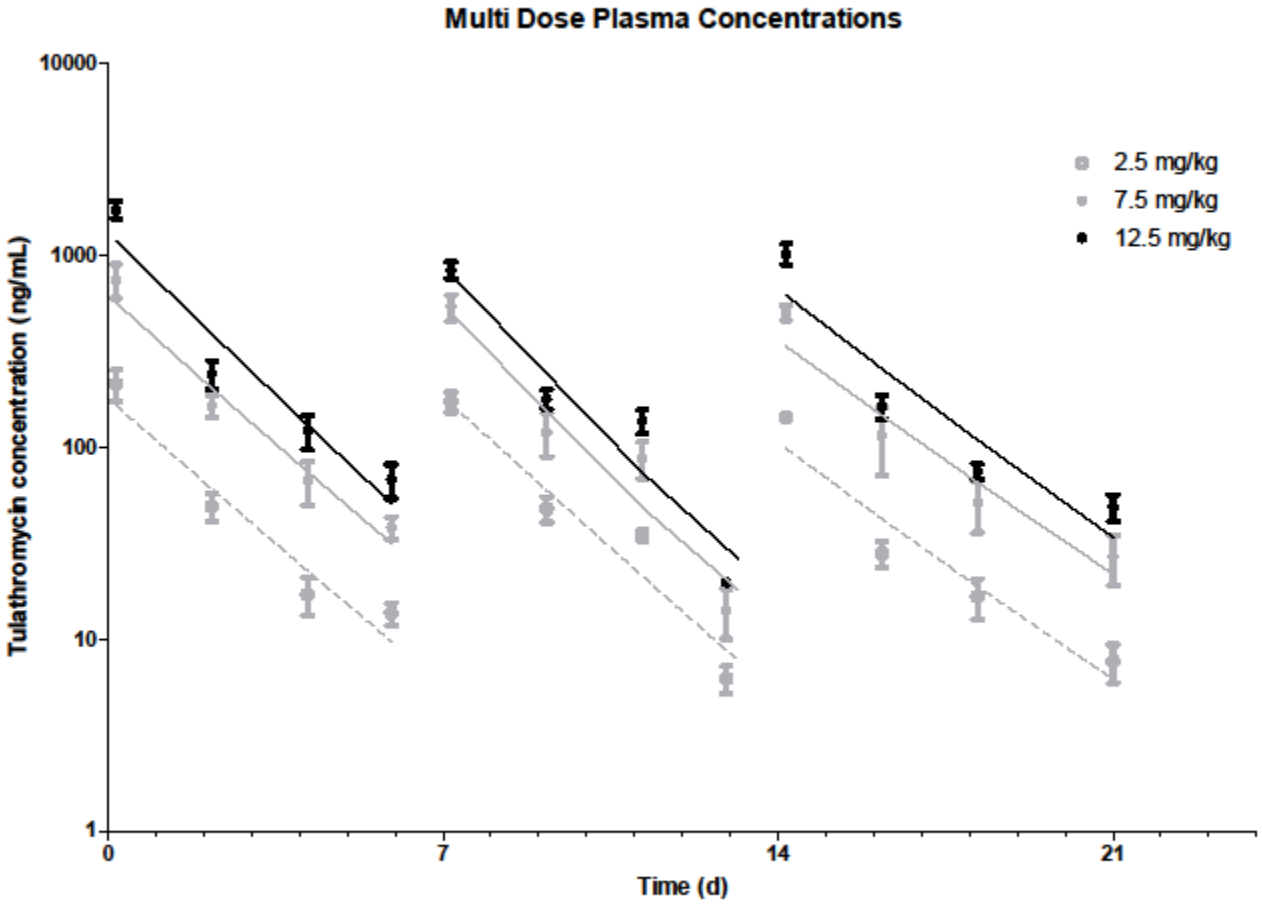

Figure 4.2. Mean ± SD of plasma concentrations from kid goats administered 3 repeated injections of 2.5, 7.5 or 12.5 mg/kg tulathromycin at 7 day intervals. The predicted plasma concentrations for the terminal phases estimated from a noncompartmental analysis of the data are plotted for each injection at each dose level. The parameters from the NCA are given in Table 3.

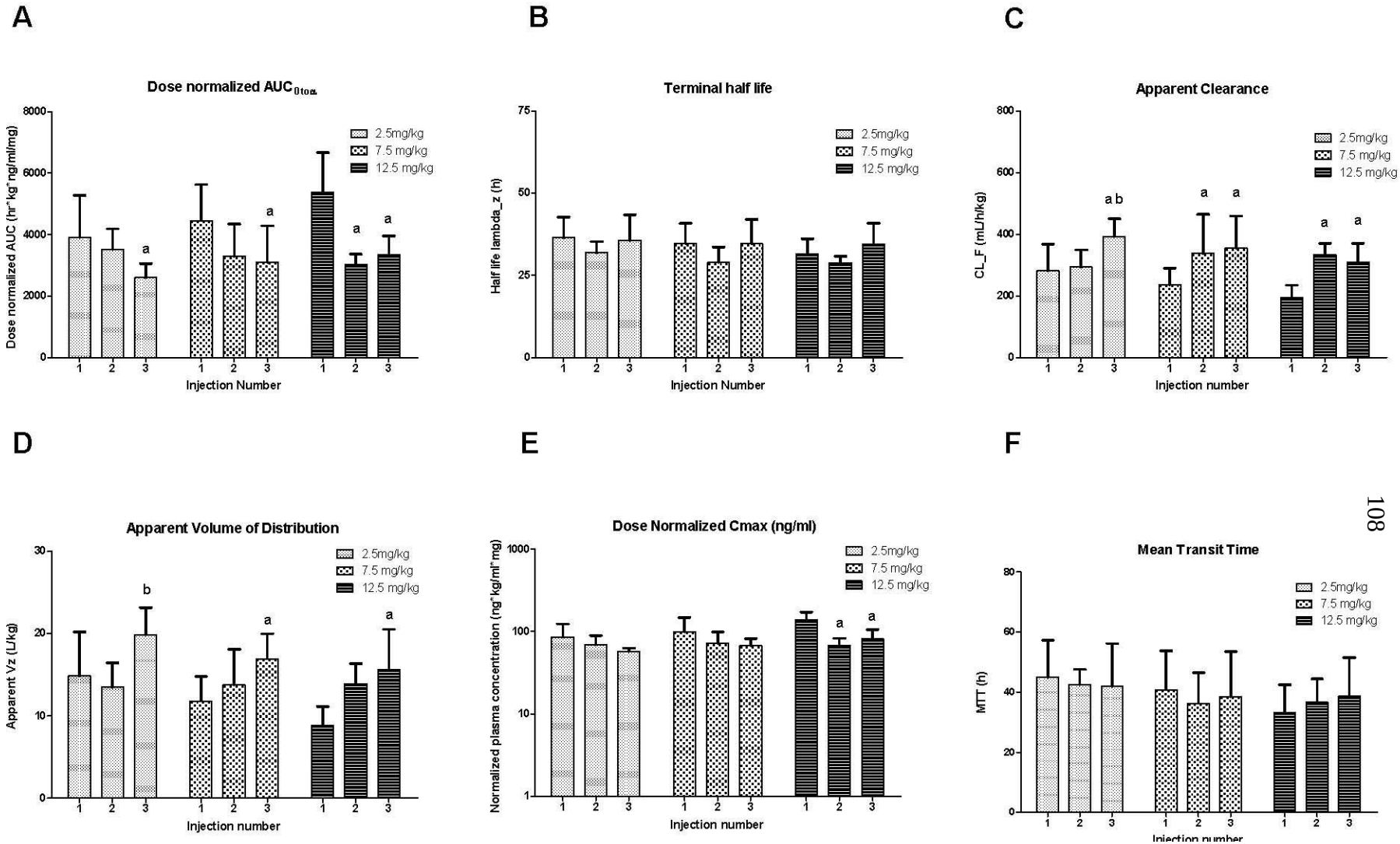

Figure 4.3. Mean ± SD of NCA parameters estimated from kid goats from multi-dose administration of 2.5, 7.5, or 12.5 mg/kg tulathromycin including A) Dose-normalized $AUC2_{\infty}$; B) terminal half life($t_{1/2}$); C) apparent clearance (CL/F); D) apparent volume of distribution (V/F); E) dose-normalized C_{max}; and F) Mean Transit Time (MTT). The parameters were analyzed by a 2 way ANOVA at $P < 0.05$ with a Bonferroni post hoc test ($P < 0.05$). Bars marked with [a] indicate a statistically significant difference ($P < 0.05$) from injection 1, and [b] indicates a statistically significant difference ($P < 0.05$) from injection 2.

CHAPTER 5. ANTIMICROBIAL SUSCEPTIBILITY PATTERNS AND SENSITIVITY TO TULATHROMYCIN IN GOAT RESPIRATORY BACTERIAL ISOLATES

A paper to be submitted to the *Journal of the American Veterinary Medical Association*

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Abstract

Bacterial pneumonia is a common and often life-threatening respiratory problem in both meat and dairy goats. Options for approved antibiotic therapy in goats to combat these bacterial infections are severely limited and frequently drugs must be used in an extra-label manner. Tulathromycin, a triamilide macrolide antimicrobial drug shown to be effective against swine and cattle respiratory bacterial agents, has been identified as a potentially useful drug in caprines. The present study was conducted to determine the susceptibility of recognized bacterial respiratory pathogens to commonly prescribed antimicrobials, with a particular emphasis on the efficacy of tulathromycin against these agents. Minimum inhibitory concentration (MIC) testing using microbroth dilution was performed on a collection of forty-five *Mannheimia haemolytica*, eleven *Pasteurella multocida*, and eleven *Bibersteinia trehalosi* isolates from the lungs of goats with clinical pneumonia. To further characterize efficacy of tulathromycin against these pathogens, minimum bactericidal

concentration (MBC) testing and kinetic killing assays were conducted. Most of the isolates were susceptible to the antimicrobials tested; however, increased resistance as demonstrated my higher MIC values was seen in all species to penicillin, in *P. multocida* to sulfadimethoxine, and in *B. trehalosi* to the tetracyclines. All isolates were susceptible to tulathromycin, which demonstrated a high killing efficiency in both bactericidal assays. Results of this study indicate that most goat pneumonic bacterial pathogens remain susceptible to commonly-prescribed antibiotics, although some evidence of resistance was seen to certain drugs; and that tulathromycin is highly effective against goat respiratory pathogens which could make it a valuable medication in this species.

Introduction

Bacterial pneumonia is a common and often life-threatening respiratory problem in both meat and dairy goats (Washburn *et al.*, 2007; Yener *et al.*, 2009). *Pasteurella multocida*, *Mannheimia haemolytica*, and *Bibersteinia trehalosi* (formerly *M. haemolytica* serotype T) often complicated by *Mycoplasma* colonization, are the most common causes of respiratory disease in sheep and goats (Ackermann & Brogden, 2000; Berge *et al.*, 2006; Brogden *et al.*, 1998; Washburn *et al.*, 2007; Zamri-Saad & Mera, 2001). Options for approved antibiotic therapy to combat these bacterial infections are severely limited (Berge *et al.*, 2006). Currently ceftiofur is the only antibiotic labeled for use in goats; however, successful treatment relies on daily therapy which may be difficult to accomplish in field situations (Fait, 2001; Fait, 2003; Washburn *et al.*, 2007; Webb *et al.*, 2004).

The Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994 permits the use of certain approved animal and human drugs in an extra-label manner under a valid veterinary-client-patient relationship (Berge *et al*., 2006; Fait, 2001; Fait, 2003; Food and Drug Administration, 1994). Prescribing veterinarians are responsible for choosing an appropriate drug, dose, and withdrawal period in the absence of label directions for the species being treated (Fajt, 2001). Minimum inhibitory concentration (MIC) data on antimicrobials most likely to be effective against known pathogenic respiratory isolates could prove useful to clinicians.

The United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) sponsors the Minor Use Animal Drug Program as the National Research Support Project-7 (NRSP-7) which provides funding to investigate drugs for potential use in minor species and for minor uses in major species (USDA National Research Support Project 7, 2007). Tulathromycin, a novel triamilide antibiotic in the macrolide class, has been shown to be safe and efficacious in the treatment of bacterial respiratory disease in cattle and swine (Evans, 2005; Nowakowski *et al*., 2004; Hart *et al*., 2006). A single injection provides seven days of therapeutic concentrations in lung tissues in these species. The drug has demonstrated efficacy against respiratory pathogens shared by cattle and goats; therefore, tulathromycin has been selected for an NRSP-7- supported project. As part of the larger supported project, the objective of this study was to identify antimicrobial susceptibility patterns in bacterial respiratory pathogens from goats with clinical pneumonia and to

specifically evaluate susceptibility of these isolates to tulathromycin using two *in vitro* methods.

Materials and methods

Bacterial isolates—The isolates in this study were collected from diagnostic laboratories from various geographic regions throughout the United States. All isolates were recovered from diseased pulmonary tissues from goats with clinical respiratory disease. Forty-five *M. haemolytica,* eleven *P. multocida,* and eleven *B. trehalosi* isolates were identified by growth and biochemical characteristics and stored in brain heart infusion (BHI) broth containing glycerol at -80^{o} C.

Blinding and Randomization—Isolates were assigned a random study designation upon arrival at the laboratory. All isolates included in the study met the source criteria—collected from diseased tissues in goats suffering from respiratory disease. All persons conducting susceptibility testing of the isolates had no knowledge about results of previous testing done on any of the isolates.

MIC testing—Susceptibility determinations were performed by microbroth dilution methods using Sensititre (Trek Diagnostic Systems, Cleveland, OH). Minimum inhibitory concentration (MIC) determinations were performed in accordance with criteria provided in M31 [A3] (Clinical Laboratory Standards Institute [CLSI], Wayne, PA). Isolates were tested against 14 antimicrobials at the listed dilutions, including ampicillin (0.25-16µg/ml), ceftiofur (0.25-8 µg/ml), chlortetracycline (0.5-8 µg/ml), danofloxacin (0.12-1 µg/ml), enrofloxacin (0.12-2 µg/ml), florfenicol (0.25-8 µg/ml), gentamicin (1-16 µg/ml), neomycin

(4-32 µg/ml), oxytetracycline (0.5-8 µg/ml), penicillin (0.12-8 µg/ml), sulfadimethoxine (256 µg/ml), trimethoprim-sulfamethoxazole (2/38 µg/ml), tulathromycin (1-64 µg/ml), and tylosin (0.5-32 µg/ml). *Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853, *Enterococcus faecalis* ATTC 29212, and *Staphylococcus aureus* ATTC 29213 were used as quality control organisms.

Bacterial isolates were sub-cultured on blood agar and incubated for 18-24 hours at 35˚C. Each isolate was suspended in phosphate-buffered saline (PBS) to a concentration equivalent to a 0.5 McFarland standard and 10µl of this standard was added to 10ml of broth for antimicrobial susceptibility plate inoculation. Cation-adusted Mueller-Hinton broth containing 100µl of fetal calf serum was used for *P. multocida* testing; Cation-adusted Mueller-Hinton broth containing lysed horse blood was used for *M. haemolytica* and *B. trehalosi* testing. Susceptibility plates were incubated for 18 hours at 35˚C and observed for visible growth. The MIC was determined as the lowest concentration of antimicrobial that prevented visible growth. All isolates were tested in duplicate on different days; isolates with any discrepancy in tulathromycin MIC were tested in triplicate so a consensus MIC value could be obtained.

In order to assess bactericidal effects of tulathromycin, an aliquot of each MIC sample well containing a dilution of tulathromycin was plated onto media without antimicrobial and incubated at 37˚C overnight to determine if bacterial growth had been inhibited or if bactericidal effects could be seen with the given concentration of tulathromycin (MBC_{100}).

Statistical analysis—MIC results were stratified for each species of bacteria and summarized; mode value and range were determined for each antimicrobial. Stratified analysis with the nonparametric Kruskal-Wallis test was used to compare tulathromycin MIC's between bacterial species. Interpretations of MIC breakpoints were extrapolated based on CLSI criteria for bacterial species associated with bovine respiratory disease complex (BRDC) and were compared for each antimicrobial between the bacterial species. "Percent susceptible" was calculated as the number of isolates classified as "susceptible" divided by the total number of isolates tested and expressed as a percentage. Statistical comparisons were made of the percent susceptible for each drug between species using Fisher's exact test using SAS statistical software (version 9.1, SAS Institute, Inc., Cari, NC).

Kinetic killing assay—To further evaluate the susceptibility of these isolates to tulathromycin, kinetic killing assays were conducted to determine the percentage of bacteria killed within two hours at a given tulathromycin concentration with a goal of establishing the minimum bactericidal concentration that resulted in > 99% of bacterial die off (MBC_{99}) during this period. Since the maximum MIC concentration for all isolates was 8µg/ml, this concentration was chosen for initial kinetic killing assays. Eleven randomly selected *M. haemolytica* isolates as well as the eleven *P. multocida* and eleven *B. trehalosi* isolates were sub-cultured on blood agar and incubated for 18-24 hours at 35˚C. A heavy inoculum was made of each isolate in sterile BHI broth containing 5% serum and 0.05% yeast and placed in a shaking incubator at 37˚C and 160 RPM for 60 minutes to establish a rapidly growing bacterial suspension. A starting inoculum of 0.280 at OD_{595} for *M. haemolytica* and *P.*

multocida and 0.350 at OD_{595} for *B. trehalosi* that corresponded to 1 X 10^8-1 X 10^9 cfu/ml was incubated at 37˚C with 8 µg/ml of tulathromycin. Aliquots of this culture suspension were removed and colony count assays performed at time = 0, 30 minutes, 60 minutes, 90 minutes, and 120 minutes for each isolate. Additionally, five (three *P. multocida* and two *B. trehalosi*) isolates that had <99% bacterial kill at 2 hours when tested at 8 µg/ml were tested against a tulathromycin suspension of 16 µg/ml with the same collection time points.

Quality assurance was provided in accordance with Good Laboratory Practices (21 CFR Part 58).

Results

Results of univariate analysis of MIC values for each antimicrobial and bacterial species are listed in Table 5.1. Most isolates demonstrated low MIC values against antimicrobials tested; however, a few isolates had values at the high end of the tested range. Differences between species in MIC value were detected for ampicillin (between all species), chlortetracycline (between all species), danofloxacin (between *B. trehalosi* and the other species), florfenicol (between all species), gentamicin (between *P. multocida* and the other species), neomycin (between *P. multocida* and the other species), oxytetracycline (between all species), penicillin (between all species), tulathromycin (between all species), and tylosin (between *P. multocida* and the other species). No isolates that were tested in triplicate had an MIC result that varied by more than one dilution in repeat testing.

Utilizing the susceptibility definitions established for these bacteria in bovine respiratory disease, interpretations were determined for these isolates and are listed in Table 5.2. Most

of the caprine isolates showed high levels of susceptibility to most of the antimicrobials tested. Notable exceptions include penicillin (*M. haemolytica*, 13.3% susceptible; *P. multocida*, 72.7% susceptible; *B. trehalosi, 0% susceptible*); tetracycline (*B. trehalosi,* 45.5% susceptible; and sulfadimethoxine (*P. multocida,* 45.5% susceptible).

One hundred percent of these isolates were classified as susceptible to tulathromycin. Comparison of results of MIC and MBC_{100} testing of tulathromycin in each bacterial species is shown in Figure 5.1. In all three bacterial species, many of the isolates had MIC and MBC_{100} values that were identical (*M. haemolytica* 34/45, 53.3%; *P. multocida* 9/11, 81.8%; *B. trehalosi* 5/11, 45.5%).

Kinetic assays further demonstrated that 100% of *M. haemolytica* isolates tested had MBC_{99} at 8 μg/ml; 72.7 (8/11) of *P. multocida* isolates had MBC_{99} at 8 μg/ml with an additional two isolates (18.2%) having a MBC_{99} at 16 μg/ml; and 63.6% (7/11) and 27.3% (3/11) of *B. trehalosi* isolates with a MBC_{99} of 8 μg/ml and 16 μg/ml, respectively. The remaining *B. trehaolosi* isolate had a 98.0% decrease in colony counts after two hours of contact with 8 μg/ml of tulathromycin while the last *P. multocida* isolate showed a 96.7% decrease with 16 μg/ml of tulathromycin suspension over this same time period. Results of kinetic assays are summarized in Figure 5.2.

Discussion

Although no CLSI guidelines for MIC breakpoints have been established for bacterial species from goats, application of susceptibility guidelines for cattle pathogens to the same agents recovered from goats suggest that these bacteria remain relatively susceptible to most

commonly-used antimicrobials in veterinary medicine. MIC values showed some differences between and within bacterial species, but interpretations of susceptibility versus resistance were very similar. This indicates that mechanisms of antimicrobial resistance do not appear to be widespread in goat respiratory pathogens. Interestingly, *B. trehalosi* isolates demonstrated a greater resistance to the tetracycline drugs tested than the other bacteria species while *P. multocida* showed marked resistance to sulfadimethoxine. High level resistance to penicillin was seen in all three species.

Macrolide antimicrobials are characterized by rapid dissemination out of plasma and into lung, making them useful in the treatment of bacterial pneumonia (Williams & Sefton, 1993). Newer macrolides such as tulathromycin have been developed that provide improved lung tissue penetration and extended half lives.(Benchaoui *et al*., 2004; Evans, 2005) Bactericidal antimicrobials have MIC values that are very close to MBC values, while bacteriostatic drugs have MBC that are much greater than MIC values (Andrews *et al.*, 2001). In the present study, 63% of all isolates had MBC that were equal to their MIC for tulathromycin, indicating that tulathromycin demonstrates bactericidal activity. Results of the kinetic killing assays further support the efficacy of tulathromycin against respiratory bacterial pathogens.

Bactericidal compounds have been defined by the ability to provide a 99% reduction in colony number in an *in vitro* assay (Dorfman *et al.*, 2008). While MIC and MBC_{100} assays evaluate the effect of a drug over a 18-24 hour incubation period, kinetic kill assays more directly measure the degree of bacterial killing at given concentrations of antimicrobial over a short time period (Dorfman *et al.*, 2008). Rapid killing of caprine respiratory pathogens

seen in the kinetic kill assays of this study provided further evidence of tulathromycin efficacy against these agents.

Results of this study indicate that goat respiratory bacterial pathogens remain susceptible to the most commonly prescribed antibiotics in veterinary medicine, and that tulathromycin could be a useful antimicrobial against caprine respiratory pathogens. The benefits of extended therapeutic activity from a single injection could prove valuable in this species.

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Table 5.1. Summary statistics of MIC values and *P*-values for differences in antimicrobial susceptibility between bacterial species for *M. haemolytica*, *P. multocida*, and *B. trehalosi* isolates collected from goat tissues against tested antimicrobials.

	M. haemolytica		*P. multocida*		*B. trehalosi*		
Antimicrobial	Mode	Range	Mode	Range	Mode	Range	*P*-value
Ampicillin	< 0.25	< 0.25-0.5	< 0.25	< 0.25	0.5	< 0.25- >16	< 0.001
Ceftiofur	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	1.000
Chlortetracycline	< 0.5	< 0.5-8	< 0.5	< 0.5	< 0.5-8	< 0.5-8	< 0.001
Danofloxacin	< 0.12	< 0.12-0.25	< 0.12	< 0.12	< 0.12	< 0.12- >1	0.020
Enrofloxacin	< 0.12	< 0.12-0.5	< 0.12	< 0.12	< 0.12	< 0.12-1	0.389
Florfenicol	1	0.5-1	< 0.25	< 0.25-0.5	0.5	0.5-1	< 0.001
Gentamicin	2	2-4	< 1	< 1-2	2	< 1-2	< 0.001
Neomycin	< 4	< 4-8	< 4	< 4	8	< 4-16	0.005
Oxytetracycline	< 0.5	< 0.5->8	< 0.5	< 0.5-2	8	< 0.5- >8	< 0.001
Penicillin	0.5	< 0.12->8	< 0.12	< 0.12-0.5	1	0.5- >8	< 0.001
Sulfadimethoxine	< 256	< 256->256	< 256	< 256->256	<256	< 256	0.231
Trimethoprim-Sulfamethoxazole	< 2	< 2	< 2	< 2	< 2	< 2	1.000
Tulathromycin	4	2-8	2	< 1-4	4	2-4	< 0.001
Tylosin	32	32->32	32	16-32	> 32	32->32	< 0.001

Table 5.2. Percent of isolates interpreted as susceptible and *P*-value for statistical differences between isolates of *M. haemolytica*, *P. multocida*, and *B. trehalosi* collected from goats with pneumonia for individual antibiotics.

	M. haemolytica n = 45	*P. multocida* n = 11	*B. trehalosi* n = 11	*P*-value
Ampicillin	97.8	100.0	90.1	0.224
Ceftiofur	100.0	100.0	100.0	1.000
Chlortetracycline	93.3	100.0	45.5	< 0.001
Danofloxacin	100.0	100.0	90.1	0.162
Enrofloxacin	97.8	100.0	90.1	0.224
Florfenicol	100.0	100.0	100.0	1.000
Gentamicin	100.0	100.0	100.0	1.000
Neomycin	97.8	100.0	90.1	0.224
Oxytetracycline	93.3	100.0	45.5	< 0.001
Penicillin	13.3	72.7	0	< 0.001
Sulfadimethoxine	91.1	45.5	10.0.0	0.0002
Trimethoprim/ sulfamethoxazole	100.0	100.0	100.0	1.000
Tulathromycin	00.0	100.0	100.0	1.000
Tylosin	0	0	0	1.000

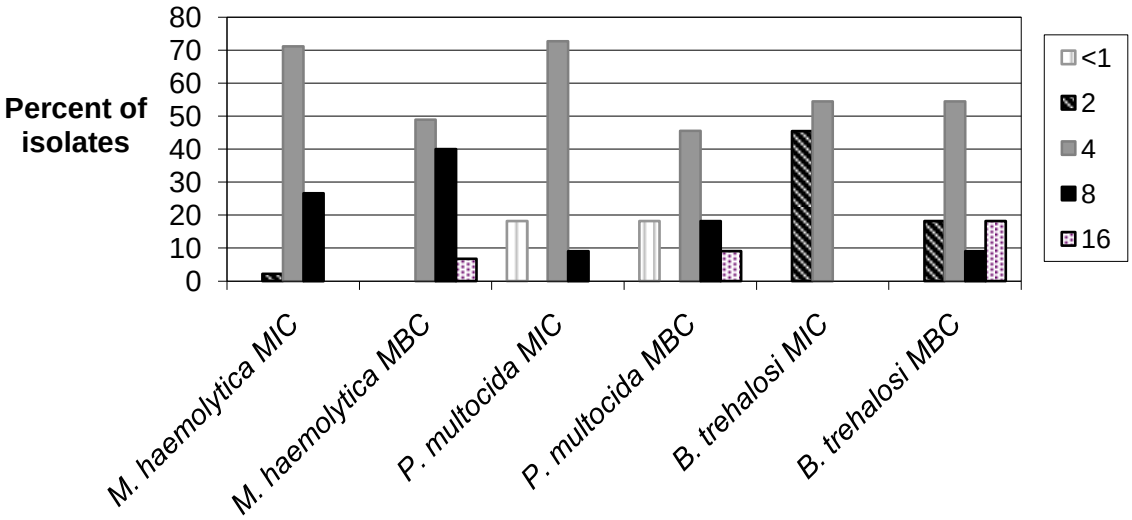

Figure 5.1. Percent of isolates with minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for tulathromycin against *M. haemolytica*, *P. multocida,* and *B. trehalosi* isolates recovered from goats with clinical pneumonia.

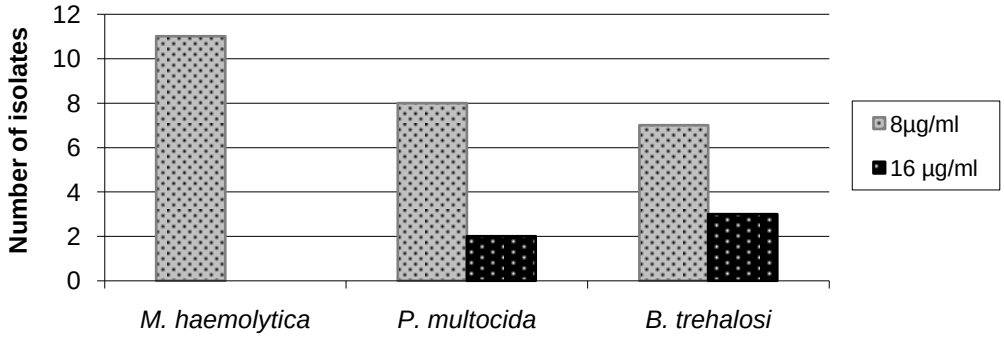

Figure 5.2. Results of kinetic killing assays evaluating the tulathromycin concentration associated with 99% bacterial killing (MBC_{99}) in the first two hours of exposure for *M. haemolytica*, *P. multocida*, and *B. trehalosi* isolates recovered from goats with clinical pneumonia.

CHAPTER 6. CONCLUSIONS AND FUTURE RESEARCH

This manuscript describes the results of studies conducted in order to support the FDA requirements for tulathromycin approval to treat bacterial pneumonia in goats. The TAS study findings were consistent with those of Washburn et al., (2007), and determined that no detrimental effects could be attributed to tulathromycin administration, even at five times the label dose and three times the label duration for cattle and swine**.** Our study was performed in very young goat kids rather than in adult goats under the assumption that the younger animals would be most likely to manifest adverse effects due to tulathromycin overdose**.** Our study took safety evaluation further to ensure no detrimental effects associated with tulathromycin could be detected on gross necropsy, histologic exam or clinical pathology. For human food safety evaluation, tissue residue analysis was performed on three separate groups of goats: the goats from the TAS study; goats used in a preliminary study to ensure that adequate pharmacokinetic and tissue collection procedures could be established for subsequent large studies; and market-age goats that would be most likely to enter the human food chain. The tissue residue studies provide information to support the establishment of a withdrawal period following administration of tulathromycin by a subcutaneous route. Since methods of assessing efficacy in cattle and swine were not amenable to goat production systems, alternative parameters of plasma tulathromycin analysis, lung tissue tulathromycin analysis, and *in vitro* bacterial susceptibility to tulathromycin were used to establish AUC/MIC ratios against the most commonly recovered bacterial pathogens in goats.

While adequate numbers of *M. haemolytica* isolates were collected to meet the minimum FDA requirements, only 11 *P. multocida* and 11 *B. trehalosi* isolates, and 9 *Mycoplasma* isolates were located. Future work will be aimed at identifying additional isolates of these species for testing and to creating a tulathromycin assay that can be used to establish MIC values for *Mycoplasma* isolates.

Additional studies could be conducted that measure the tulathromycin concentrations in specific aspects of the lung tissue. Alveolar macrophages have been used as a representative cell type recruited during the immune response to a bacterial infection. Evaluation of the tulathromycin concentration in this specific cell type may provide more precise measurements of the drug available at the site of bacterial infections in the lung; however, this type of evaluation would not account for tulathromycin present in other immune cells such as neutrophils nor does it provide information about drug presence in extracellular spaces such as interstitial fluid or endothelial secretions. Serial collection of pulmonary macrophages retrieved via broncho-alveolar lavage sampling for tulathromycin analysis may not yield an accurate representation of drug concentrations since the collection process will incite an influx of inflammatory cells, particularly macrophages.

In cattle and swine studies, lung tissue homogenates were used to assess total tulathromycin concentrations. Similar procedures were utilized in these goat studies to facilitate comparison of results and demonstrated that total drug exposure was similar between the species. Given the pharmacokinetic and pharmacodynamic similarities of tulathromycin between species, one could reasonably predict that tulathromycin should be

just as efficacious in goats as it has proven to be in cattle and swine. Reports from the field where tulathromycin has been used in caprines under veterinary prescription according to AMDUCA regulations also indicate that tulathromycin is proving to be a valuable antimicrobial for use against bacterial respiratory infections.

Information collected in the present studies can also be used to provide predictive models for use in extrapolation to other species, particularly other species such as sheep, llamas, alpacas, and deer. While extrapolation of drug behavior between species is less desirable than actual studies in the target species, it can provide guidance for veterinarians who prescribe antimicrobials under AMDUCA requirements to other minor species. Data from the studies in caprines may also be useful in designing protocols for studies to be conducted in other species and facilitate adherence to FDA requirements for approval of tulathromycin in these species.

The FDA CVM is responsible for ensuring the safety and efficacy of all drugs labeled for use in animals in the United States. Requirements maintained by the FDA for approval are justifiably strict, specifying that a given drug be evaluated in a specific species to treat an individual condition or set of conditions. Only by utilizing such defined criteria and maintaining rigorous standards for evaluation of potential medications can the FDA ensure that a product has been adequately investigated for safety, efficacy, and potential effects on humans before it is released onto the market. While the AMDUCA gives veterinarians the authority to prescribe a drug when deemed medically necessary under a valid veterinary-client-patient relationship, this directive places the responsibility for any effects of this drug

on the veterinarian. Without adequate data from controlled scientific studies, veterinarians may prescribe compounds which produce unintended consequences in animals being treated or result in undesired products entering the human food chain. Minor species or uncommon conditions present unique challenges for veterinarians. Extrapolation of data from similar species, computer simulations, and mathematical modeling can be useful in guiding veterinarians in drug administration; however, these techniques cannot provide the same level of critical assessment that can be gained from studies evaluating a specific medication in the target species to be treated.

As more is learned about the development of antimicrobial resistance in bacterial isolates and its relationship with antibiotic resistance in human and animal pathogens, future restrictions on drug use is to be expected. Careful evaluation of risks and benefits of antimicrobial use along with critical scientific assessments of these drugs will ensure that they are prescribed in the most judicious manner while minimizing the potential for side effects. FDA guidelines are designed to provide this critical assessment and will help to ensure that only safe and effective medications with adequately-defined withdrawal periods enter the marketplace. It is to the benefit of the veterinary profession as well as to agricultural industries to ensure that these strict regulations are respected and adhered to in order to ensure that high quality animal-derived products without drug residues enter human product markets.

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